VO NUMBER 2

OLOGY

LABORA AL

Fairchild Culture [illegible] A—a superior culture [illegible] laboratory technic.

Pepsin, Fairchild—[illegible] and anti-toxin laboratory.

Trypsin, Fairchild—[illegible] other enzymes and constituents [illegible] product of [illegible] labelled "trypsin."

Bile Salts, Fairchild—[illegible] sodium glycocholate and taurocholate in their native association, separated from [illegible] ox gall.

Holadin—the active [illegible] with associated [illegible] with [illegible] subject in therapeutics; especially [illegible]

Holadin and Bile [illegible] between the bile and the pancreatic [illegible] others), and of [illegible]

Gastrone—non-[illegible] [illegible] mucous membranes; [illegible] and inorganic [illegible]

Enzymes—for [illegible] meat of lean [illegible] acids, etc., [illegible] curdible [illegible] intensifying [illegible] freely diffusible [illegible] simple dilution [illegible]

Fairchil[illegible]

Specialists in [illegible]

JOURNAL OF BACTERIOLOGY

OFFICIAL ORGAN OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

DEVOTED TO THE ADVANCEMENT AND DISSEMINATION OF KNOWLEDGE IN REGARD TO THE BACTERIA AND OTHER MICRO-ORGANISMS

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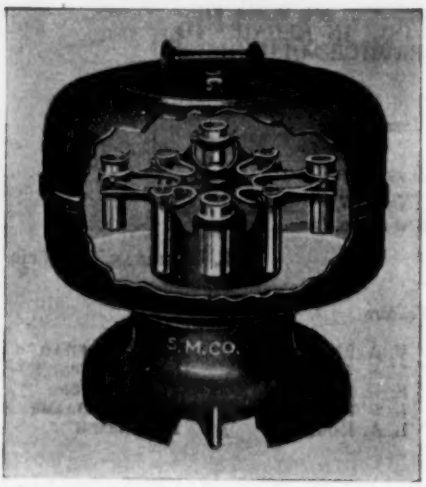

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THE SCIENCE OF BACTERIOLOGY AND ITS RELATION TO OTHER SCIENCES[1]

LEO F. RETTGER

Yale University

Bacteriology is a child of many adoptions, ever precocious, but not yet fully mature. Born with a definite mission to serve and to save, it has re-created pathology, given inspiration and new life to botany and zoology, contributed generously of its substance to agriculture and home economics, and lent itself as the framework around which modern hygiene and preventive medicine have been built. Yet all the while it has conducted itself in competent hands as a pure science.

Bacteriology has, however, been the victim of gross paternalism by those sciences which it has come to redeem. Botany in particular has long laid a claim to it; but it is to pathology that it has been holding itself in bondage, as the result perhaps of their close affiliation since the early days of Pasteur, Lister and Koch. Like the sturdy youth who has long passed his majority, but who suddenly realizes his own powers to conduct affairs for himself or to claim due recompense for his arduous labors in support of a stern father or guardian, bacteriology must and will emerge from its servile state.

Nor has the bacteriologist himself been entirely free from blame. In spite of abundant opportunities for the most valuable scientific researches, and for the promulgation and application of important principles and relationships, faulty and incomplete preparation have too often taken the place of long and thorough training in the subject. Many positions have been, and are, even

[1] Address of the President of the Society of American Bacteriologists, delivered at Nineteenth Annual Meeting, Washington, December 28, 1917.

today, filled by persons whose preparation has been sadly inadequate. How often the newly appointed bacteriologist to a municipal or state board of health is a physician whose complete knowledge of bacteriology has been acquired in the short period of eight or ten weeks of a regular medical school curriculum!

The bacteriologist can not be made in a day. Dr. Moore in his admirable presidential address dwelt upon the need of thorough training in bacteriology. No wonder that he was dismayed at the failure to evolve a finished product at the end of the regular college course. How can a bacteriologist be made in three or six months, when it takes at least as many years to produce a chemist, a physicist or an electrical engineer? It is my firm conviction that we cause irreparable injury to our science when we recommend for positions men or women who have not had the same advanced training that is as a rule required of the chemist, for example. They must be able to do more than to pour gelatin and agar plates and to count colonies.

The trained bacteriologist of the future will need a deep and broad foundation upon which he is to erect his superstructure. A knowledge of general biology and chemistry will be as essential for him as arithmetic and algebra are for the man who is entering upon a course in higher mathematics. To this must be added elementary physics and a reading knowledge of French and German, at least.

It is indeed pathetic to see young men or women of undoubted ability and promise apply for admission into the graduate department of a university and to see them denied admission because of inadequate preliminary training. Pathetic, I say, because of the sudden realization that comes over such earnest seekers for the truth, a realization that they are unfit in spite of their natural ability.

With a general college training covering the above subjects, the student who has chosen bacteriology as his chief field of activity and life work has a wide choice of closely allied subjects, as for example biochemistry, protozoology, parasitology, pathology, sanitary chemical analysis, sanitary engineering, and hygiene and public health. He must at no time lose sight of

the disciplinary value of this or that subject, but his main interest should be in those channels which will aid him materially in his mastery of bacteriology as a real science, and in its many important relationships.

Bacteriology differs from all other sciences. Its technique and methods of experimentation and control are its own. It has its own problems. Yet like other sciences, and even to a greater degree, it must borrow from, and give to, other fields of study. Its relations and applications to the various industries, to hygiene, medicine, etc., are many; but at the very foundation its principles are as thoroughly scientific as those of any other branch of knowledge. Coulter has said that what determines a subject as a real science is not only the subject matter itself but also the purpose with which it is pursued.

To study bacteria as the ornithologist does birds, or the geologist, rock formation—in other words, to learn nature's secret in so far as it is revealed by this large group of living organisms—is indeed as truly a scientific inquiry as the most profound investigation into the structure of the protein molecule. Nor need such a study be limited to organisms which are of no interest to the student of medicine. The general student of bacteriology is just as much entitled to a knowledge of the typhoid bacillus in its relation to environment and natural habitat as he is to a full understanding of the characters of *Bacillus subtilis*, what its common places of residence are, and what its economic rôle may be.

Bacteriology has too wide a range of activity and influence to confine it to the usual Medical School curriculum; and like physics, geology and biochemistry, it requires an academic or university atmosphere. To those who are in quest of scientific pursuits it presents many problems of profound interest, as for example those of biological classification, variation, cell growth and metabolism, the response of microorganisms to stimuli, enzyme action, organic synthesis and decay, etc. In fact the very problems of life's origin and of death belong as much to the realm of bacteriology as to any other science. There is no necessity of dwelling here on its many practical applications, which need not be any the less scientific.

If there is any one related science which, to the bacteriologist, is more important than any other, it is chemistry. It is indeed unfortunate that there are few chemists who are also bacteriologists. It is to be regretted also that comparatively few bacteriologists are familiar with the principles of chemistry. The latter assertion is well borne out by the failure of systematists to evolve even moderately successful classification schemes, and by the action of committees on standard methods whose choice of standard methods has not always been the wisest. It required a Jensen to show the way to a newer and more rational system of bacterial classification; and it was not until physical chemists came to our aid that a scientific and practical method of determining the H ion concentration of a culture medium was supplied. The study of antiseptics and chemical disinfectants has received a new impetus through the work of Chick and Martin (1908) and the recent researches of Dakin (1916) and his associates.

Pasteur's victory over Liebig in their noted controversy on the exact relationship of the yeast cell to fermentation was a victory for the biologist. Buchner's researches on the enzymes of yeast somewhat reversed the tables again, and today we are more and more being led to believe that practically all transformation of bacterial substrates is brought about through the immediate agency of an enzyme, whatever that may be.

Exhaustive chemical investigations into the composition of bacteria and their multitudinous products are necessary before we can acquire an understanding as to what bacteria in reality are and what they can do. The investigations of Von Nencki and his pupils promised a wide development of this field. Others have made most valuable contributions, as for example Brieger, Kühne, and the Martins. In more recent times, the researches of Tamura on the chemistry of bacteria, are of particular interest; also the work of Armand-Delille (1913) and his associates on the significance of amino acids and di-amines in culture media, and the investigations of others (Sasaki, (1912) Otsuka (1916), etc.) on the possible rôle of polypeptides in bacterial nutrition.

Bacterial nutrition is essentially a biochemical problem, but should make an emphatic appeal to the bacteriologist. It has as yet been but little explored. The impelling need for appropriate synthetic media in the biometric or quantitative study of physiological activities of bacteria remains today practically unsupplied. We are as yet in the dark regarding the real food requirements of bacteria.

Until very recently it was a common assumption that practically every sort of organic matter is food for bacteria. Today we are made to realize that the problem of dietetics in bacteriology is as real as in animal physiology. Indeed we are observing more and more that the physiology of bacteria and of animal organs is not so very unlike. The processes involved in the pancreatic digestion of protein are in a large measure reproduced by the proteolytic enzymes of organisms of the *B. subtilis* and *Proteus vulgaris* types, certainly by the organisms of putrefaction. Bacterial enzymes are able to bring about the rapid cleavage of true proteins with the production of essentially the same decomposition products as those which are formed in ordinary tryptic digestion, namely albumoses, peptones and polypeptides, various amino acids (leucine, tyrosine, etc.) and tryptophane. Certain products are, of course, characteristic of the bacterial cell, as for instance indol, the aromatic oxyacids, and the ultimate decomposition products, ammonia, hydrogen, methane, carbon dioxide, etc.

The problems of assimilation of food are essentially the same for the bacterial and animal cell. Foods must be of very simple composition in order that they may be utilized for cell growth. This was demonstrated but a few years ago by Loewi, Abderhalden and others in animal physiology. In the words of Abderhalden, "It is as essential to break down complex nitrogenous food substances into their simple components, before they can be utilized, as it is to reduce the walls of an old church brick by brick before they can be made over into a modern schoolhouse." Recent investigations in our laboratory have impressed most vividly upon us the same fact with reference to bacterial nutrition.

We have been led through oft-repeated experiments on animal and vegetable proteins to conclude that all bacteria are unable to derive nourishment from native proteins, and that in a medium in which there is no other possible source of nitrogen than the proteins themselves they will thrive no better than in a chemically pure saline solution. When cleavage-producing agents, like a proteolytic enzyme, are present, the complex protein molecules are broken up and, at least in part, made available for cell nutrition.

We have been able to show also that albumoses and the more complex peptone fractions of Witte's and other commercial peptones are like stone to the bacterial cell. Not only are bacteria unable to utilize these substances without preliminary cleavage or separation into their constituent parts, but many organisms are without the ability to produce the enzymes necessary to prepare these complex organic substances for cell nutrition. The Coli-typhi-dysenteriae group furnishes notable examples of such organisms. With but few exceptions there appears to be a correlation between this property and that of gelatin liquefaction.

The question may well be asked, "Is not bacterial action essentially synthetic?" The limited knowledge at our command would, I believe, dictate the answer "Yes." If this question can be answered in the affirmative, our present system of media-making and culture study will need thorough revision; but before there can be much intelligent revision, further and more far-reaching investigations into the real requirements of a bacterial culture medium are indeed necessary.

We are told again and again that the so-called "parasitic" or "pathogenic" bacteria require a medium which simulates that of the natural animal host of these organisms. As a consequence of this prevailing idea fresh blood, blood agar, blood serum and ascitic fluid bouillon and agar, are regarded as indispensable in the isolation and cultivation of certain of the pathogens. In many instances sterilization by heat is avoided, in order that the natural nutrient properties may be preserved.

That these animal fluid media are valuable, and, in some instances, to all appearances necessary, cannot be denied. The common notion, however, that the peculiar nutrient properties are inseparably linked with the proteins as such, and that the value of the media depends upon these proteins, has little to support it. It is far more probable that the food-giving substances in the body fluids or tissues are nitrogenous substances of very simple constitution, perhaps of the nature of amines and amino acids. It must be assumed that in the profound chemical changes which take place in these fluids in the animal body, and in the process of autolysis after death or after their removal from the body, many and varied nitrogenous substances are formed which possess different degrees of stability. Some of the products are without doubt easily transformed into more stable substances, but, because of the extraordinary ease with which they are transformed, serve as excellent grist for bacteria, very much in the same way that a high grade of gasolene explodes more readily in the gasolene motor than one of much lower boiling point. Others, while less readily appropriated by the bacterial cell, are sufficiently unstable to be seized and utilized. The latter may in a large measure resist short periods of heat sterilization, while the more unstable intermediate products are destroyed in the process.

The extracts of animal organs, as well as those of some plant tissues, contain valuable nutrient material for bacteria which it is as yet impossible to supply in any medium of known chemical composition. Even commercial peptone possesses much value as an early stimulator of growth. This may be demonstrated readily on the diphtheria bacillus. The bacillus of Loeffler does not adapt itself to synthetic media. If but a very small amount of commercial peptone be added to the ordinary Uschinsky medium, however, growth soon takes place. Peptone is indeed a highly complex food, but it owes its value primarily to the substances of simple constitution which it contains. A peptone prepared without the application of heat in any stage of its preparation, if this were possible, would without doubt possess still greater value as a food for bacteria.

Fresh meat extract, also, is excellent nutriment for the bacterial cell. Commercial meat extract, while more uniform in composition than the other, loses much of its richness in the long process of preparation which involves prolonged application of heat and contact with filters.

The chemical composition of these more or less unstable but highly nutritive substances is a matter of purest speculation. For want of a better name they may well be termed "vitamins" or "accessory growth factors." While some are very unstable, others perhaps will withstand a certain amount of heat, and even rapid steam sterilization. The diphtheria bacillus seems to grow about as well on serum sterilized by the ordinary intermittent method as by the process originally advocated. It is a well known fact that *Treponema pallidum* will grow in ascitic fluid medium which has been sterilized by heat, and that, therefore, extreme precaution to use absolutely uncontaminated ascitic fluid is unnecessary. That there is danger of too strong application of heat is of course not to be denied.

The occurrence of vitamins in animal fluids, and probably in vegetable tissues, and their significance in the cultivation of the meningococcus, have received considerable attention by Dorothy Lloyd (1916) in her recent cultural studies of this organism. According to these investigations, primary cultivation of the meningococcus is possible only in the presence of certain accessory growth factors which are present in blood serum and other animal fluids, and probably in vegetable tissues. These accessory bodies are moderately heat stable, and are soluble in alcohol and in water. They are rapidly absorbed from solution by filter paper, but not by glass wool. The vitamins increase the reaction velocity of the proteolytic metabolism of the meningococcus. After the first or primary artificial cultivation the meningococcus gradually becomes independent of these substances. Old laboratory strains need no vitamins so long as amino acids are present. The main food requirements are the amino acids.

Cole and Lloyd (1917) have shown the following to be important factors in the cultivation of the gonococcus. Suitable H

ion concentration, preferably $P_H = 7.6$, high concentration of amino acids, and the presence of certain special "growth hormones." The importance of blood and other animal tissues is emphasized, but it is claimed that sterilization of the medium by heat does not destroy the hormones which favor the growth of the gonococcus.

Gordon, Hine and Flack (1916) call attention to the special growth-stimulating properties of pea flour and of wheat germ extract. The former favored early growth better than the germ extract, while the wheat germ extract had the more favorable influence on the longevity of the meningococcus. Agar containing pea flour extract deteriorated on autoclaving. The vitamins were not precipitated by alcohol.

Here is a field that is full of promise to the student of bacterial nutrition. That it is fraught with difficulties, some of them perhaps insurmountable, cannot be denied. Not only is there need of improved methods of cultivation in so far as the more difficultly grown bacteria are concerned, as for example the highly specialized parasitic or pathogenic organisms, but a thorough revision all along the line is of great importance.

The isolation and the enumeration of bacteria by the usual plate method have their serious defects, in spite of the marvelous progress in bacteriology which the method of isolation on solid media devised by Koch in 1880 stimulated. And in no field is the deficiency felt more keenly than in the isolation and study of the anaerobes. It may be said, in fact, that there is as yet no accepted method of enumerating anaerobic organisms, let alone the question of isolation. The difficulty encountered is not merely one of complete anaerobiosis, but fundamentally one of nutrition. The somewhat recent methods of cultivation advocated by Tarozzi, (1905) Calderini (1909) and others, strongly support this contention, though the interpretations or explanations of the influence of the pieces of fresh tissues in ordinary bouillon, by the open tube method, have never been clarifying. Is not the favorable influence on the anaerobes one of chemical or physiological stimulation by the peculiar growth-stimulating substances which certain animal tissues possess?

With optimum conditions as to nutriment, and absence of growth-inhibiting agents, the question of anaerobiosis is perhaps not as important as it was once thought to be.

So-called "bacterial lag" has its origin, in all probability, in a paucity or temporary absence of growth-stimulating substances in the new environment to which the organisms are transferred. Penfold has shown that the size of the inoculum is a factor. With larger amounts of material transferred corresponding amounts of the "intermediate bodies" are carried over. When the amounts of inoculum are very small the individual organisms are for a time at the mercy of the new medium, the customary intermediate bodies being absent, or present in such small quantities as to be of little or no assistance. The same reasoning should hold in so far as enzymes are concerned. We have on many occasions found that by very sparse seeding of a culture medium with a vigorous proteolytic enzyme-producing organism an indefinite or indeed permanent lag period was established, when the medium was one of definitely-known chemical composition to which purified protein had been added.

According to Penfold (1914) maximum growth presupposes optimum concentration of intermediate bodies attainable by the bacteria. When the bacteria cease developing, the intermediate bodies diffuse out or disappear. The presence of inhibitory products of metabolism of the organisms must always be taken into consideration, of course. A most satisfactory culture medium, or one that will of itself practically eliminate bacterial lag, will be a medium which furnishes satisfactory substitutes for the intermediate bodies, in the form of amino acids and perhaps amines of simple composition, and also certain growth accessory substances. When such an artificial medium is available our methods of enumerating and isolating bacteria by the plating process will be attended with much greater success than they are now. Too many individual bacteria fail to develop, when planted in high dilution, for want of proper environment and assimilable food, and die from inanition.

These are problems, you say, of the bio-chemist. Quite true; but the biochemist of today must be well grounded in general

chemistry, and particularly in organic chemistry. Unless the physiological chemist is also a bacteriologist, his interest will be in the field of animal or plant physiology. The chemist must have sufficient training in bacteriology to enable him to appreciate its many-sided problems and, unless he can rely on some one else, who is competent, he must be familiar with bacteriological technique. On the other hand, the bacteriologist is required to be well enough trained in the principles of chemistry, and especially organic and physiological chemistry, to master the situations that are sure to confront him. Thorough co-operation between the bacteriologist and well-trained chemist may at times be a possible solution, but the two will, as a rule, not have the same interests.

Bacteriology is still in its infancy. Numerous phases of applied bacteriology have been developed with amazing rapidity, and have led to many and important discoveries; but bacteriology as a science among other sciences is just beginning to emerge from its peculiarly chaotic state, and promises to offer him who approaches it with the right spirit and preparation a most fertile field for study and investigation.

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METHODS OF PURE CULTURE STUDY

PRELIMINARY REPORT OF THE COMMITTEE ON THE CHART FOR IDENTIFICATION OF BACTERIAL SPECIES[1]

H. J. CONN, *Chairman*, H. A. HARDING, I. J. KLIGLER, W. D. FROST, M. J. PRUCHA, AND K. N. ATKINS

The following methods have been prepared primarily to accompany the chart recommended at the 1917 meeting for use in instruction in bacteriology. So far as they apply, they should also be used for the older chart; but many of the determinations called for by the older chart have not yet been studied by the committee, and are not included in this report. It is the desire of the committee that these methods be put in provisional use during 1918, and that they be adopted as standard methods at the 1918 meeting, after such changes have been made in them as the year's use shows to be necessary. The chairman of the committee will be glad to receive suggestions in regard to such changes. Reprints of this report may be obtained from the secretary of the society at about cost price.

PREPARATION OF MEDIA

Beef-extract broth shall have the following composition:

Beef-extract	3 grams
Peptone	5 grams
Distilled water	1000 cc.

Beef-extract agar shall be of the same composition plus the addition of 12 grams of oven-dried agar or 15 grams of commercial agar. *Beef-extract gelatin* shall be of the same composition, with the addition of 120 grams of Gold Label gelatin, or 100 grams of some brand of gelatin (such as "Bacto-gelatin," or United States Glue Company gelatin) having greater jellying

[1] Presented at the Washington meeting of the Society of American Bacteriologists, December 28, 1917.

power. These media are to be made up according to the directions given by the committees of the American Public Health Association on water analysis and on milk analysis (1916, 1917), except that white of egg maybe used for clarification if desired. It is recommended, however, that instead of using phenolphthalein in adjusting the reaction of these media the simpler and more accurate procedure be adopted of adjusting to the neutral point of brom thymol blue[2]. Bring the media to such an acidity as to turn this indicator a distinct grass-green (neither yellow green nor blue green). This color indicates approximately "true neutrality," i.e., a hydrogen-ion concentration between $P_H = 6.6$ and $P_H = 7.4$, a variation which seems to have no appreciable effect on bacterial activities. Another equally satisfactory method of adjusting media to this hydrogen-ion concentration is to bring them to such an acidity as to cause the first faint trace of permanent pink to appear with phenol red.[3]

Sugar broths. Just before sterilization 1 per cent of the required carbohydrate is to be added to beef-extract broth. Otherwise proceed as for sugar-free broth. Adjust reaction with brom thymol blue or phenol red.

Plain gelatin. Proceed as for beef-extract gelatin, but omit beef-extract and peptone. Clarify with white of egg.

Nitrate broth. For routine work this medium should contain:

Peptone	1 gram
KNO_3	1 gram
Distilled water	1000 cc.

Filtering is unnecessary, and reaction requires no adjustment. Modification of this formula is necessary for some organisms (see p. 124).

Starch agar. Add 0.2 per cent of water-soluble starch to the ordinary beef-extract agar.

Indicator media. Saccharine media with some indicator to show acid production are frequently used. Litmus is the most

[2] 0.04 per cent di bromp thymol sulphonphthalein in 95 per cent alcohol.

[3] 0.02 per cent phenol sulphonphthalein in 95 per cent alcohol.

common indicator, enough of which should be added in saturated aqueous solution to give the medium a distinct blue color (taking care that the litmus solution used is not so alkaline as to alter, appreciably, the reaction of the medium). Litmus, however, does not give accurate results in terms of hydrogen ion concentration; so except for certain special purposes (see p. 124) it is recommended that brom cresol purple be used. Prepare this by dissolving 0.4 g. of di bromo ortho cresol sulphonphthalein in a minimum amount of alcohol, making up to 1 litre with water. Add 40 cc. of this solution to a litre of the medium. The blue or purple color given to media by this indicator begins to fade with the slightest production of acid in the medium and disappears completely at an acidity corresponding to $P_H = 5.0$, considerably below the curdling point of milk.

Variations of these media. For certain organisms the above formulae are not the best—many pathogenic bacteria, for instance, require more peptone than is provided in the above formula for broth, while some organisms are best studied in media of a hydrogen-ion concentration different from that recommended above. In such cases the individual investigator is free to modify them to suit his own purposes; but whenever other than these standard formulae are used, the fact should be stated on the chart. In employing a reaction other than that of neutrality it is recommended that instead of using the titrimetric method, the reaction be adjusted to some definite shade of brom cresol purple, if a more acid reaction is desired, or of phenol red if it is to be more alkaline.

Optional media. In many laboratories other media than those specifically mentioned on the chart are in general use, such as potato, blood serum, agar stabs, and so forth. Blank spaces are left on the chart for recording characteristics on any optional media.

INVIGORATION OF CULTURES

Provided a medium can be found upon which the organism to be studied grows vigorously, it should always be invigorated

before study, even though freshly isolated from its natural habitat. The procedure to employ is as follows:

Prepare duplicate sub-cultures in standard glucose broth, and on standard agar slopes, placing cultures of each at 37° and 25°C. On the basis of the resulting growth the organism falls into one of the following series:

Series I. Organisms which produce good growth (surface growth, distinct turbidity, or heavy precipitate) in twenty-four hours at 37° in glucose broth.

Series II. Organisms which do not produce good growth in twenty-four hours as above, but do in forty-eight hours at 25° in glucose broth.

Series III. Organisms which do not grow well in glucose broth but do produce good growth on the surface of agar in twenty-four hours at 37°.

Series IV. Organisms excluded from the above groups but which produce good growth on the surface of agar in forty-eight hours at 25°.

Record the series number on the chart at the proper place and proceed with the invigoration by inoculating into another tube of glucose broth for organisms of series I and II, or of standard agar for organisms of series III and IV. Incubate this tube at the temperature, and for the time, called for by the series in which it belongs; then transfer from this tube to a third tube and incubate as before. From this third culture make a gelatin or agar plate and incubate at the temperature previously used until colonies of sufficient size for isolation are obtained. Transfer from a typical colony to one or more agar slants and incubate one day at 37° or for two days at 25° according to the temperature relation of the organism studied.

In case the organism does not produce vigorous growth on either of these media at either temperature, it should be invigorated with any medium and at any temperature known to be adapted to its growth. Under such circumstances invigorate by the procedure just outlined but using the medium and temperature found most favorable for the organism in question, recording on the chart the method of invigoration adopted. If no

conditions are known under which the organism in question produces vigorous growth, it should be studied without preliminary cultivation as soon as possible after isolation from its natural habitat. Such an organism is not likely to give good growth on any ordinary media, and the results of the study called for by the chart will have little significance.

STUDY OF MORPHOLOGY

The routine study of morphology should be from dried preparations, stained with fuchsin, methylen blue, or gentian violet. Preparations to show the vegetative cells should be made, preferably, from agar slant cultures, from a few hours to two days old, according to the rapidity of growth. The medium and temperature used and the age of the culture should be recorded.

Motility. Hanging-drop preparations of young broth or agar cultures should be examined for motility. If motile, microscopic preparations should be made to show the arrangement of the flagella, using any of the ordinary methods of flagella staining with which the student can obtain good success. Even if motility is not observed in hanging-drop, it is wise to attempt a flagella stain, because motile organisms often lose their motility under the conditions of observation. Even negative results from both hanging-drop preparation and flagella stain do not absolutely prove that the organism is immotile.

Presence of spores. Routine examinations for spores should be made on stained, dried preparations from agar slant cultures a week old. Stain with methylen blue. Vegetative forms take the stain, but spores do not. In most cases there will be no trouble in finding spores if the organism produces them. All rather larger rods however, (0.8 micron or more in diameter) should be regarded as possible spore-producers, even though microscopic examination does not show spores. Such bacteria should be mixed with sterile water and heated to 85°C. for ten minutes; if still alive, spores may be regarded as unquestionably present. Also make repeated transfers of the culture onto agar

and examine at various ages. A culture of a large rod should not be recorded as a non-spore-former unless all these tests are negative.

Capsules. An organism should not be recorded as having capsules unless they have been actually stained by one of the methods of capsule-staining described in bacteriological text books.

Irregular forms. Forms that differ from the typical shape for the organism (i.e., "involution forms," etc.) such as branching forms, clubs, spindles or filaments should be noted and sketched.

Special stains. In making the Gram or Neisser stain and in testing the acid-fast properties of the organism, directions given in any reliable laboratory manual may be followed.

Sketches. Drawings of all the morphological characteristics should be made on the blank spaces on the chart to the right of the descriptions. Both typical and atypical forms should be sketched, using care to designate which are typical.

CULTURAL CHARACTERISTICS

Cultures for the study of cultural characteristics should be incubated at 37°C. in case of organisms of series I and III, and at 25° in case of organisms of series II and IV, except that gelatin cultures should be incubated at 20°. Room temperature may be used in place of 25° at certain seasons of the year; but if a minimum thermometer shows that the temperature falls below 22° during the course of the work, note should be made of the fact. On the day when good growth first appears the proper descriptive terms on the card should be underlined; after subsequent study, the changes should be noted in the space provided, and sketches of the different stages should be made.

PHYSIOLOGY

Liquefaction of gelatin. Old method. The method in most common use is to hold gelatin stab cultures six weeks at 20°C. Plain gelatin should be used.

Provisional method. It is recommended that the following method proposed by Rothberg (1917) be put in provisional use until experience shows its value. It is designed to distinguish "true liquefiers" (organisms producing ecto-enzymes) from the organisms that produce endo-enzymes of proteolytic action that are released from the cell after death and cause liquefaction of the gelatin if incubated for the long period mentioned above. The method is to give the organism a preliminary cultivation for eighteen to forty-eight hours (according to its rapidity of growth) in a 1 per cent solution of gelatin at 25° or 37° according to its temperature relations; then inoculate on surface of gelatin in test tube and incubate 15 days at 20°.

Relation to free oxygen. Provisional method. Determine by noting the presence or absence of growth in open and closed arm, respectively, of fermentation tubes containing glucose broth. Care must be taken to use fermentation tubes from which the dissolved oxygen has been recently driven off by heating. In case of gas production, this test is of comparatively little value, because bubbles of gas may carry the sediment up with them; hence if an organism produces gas from glucose, the test should, if possible, be made in the presence of some other sugar which it attacks (acidifies) without gas-formation. It must be remembered, however, that even anaerobes do not grow in the absence of free oxygen except in the presence of a chemical substance (such as carbohydrate) which they are able to reduce and use as a source of oxygen.

Fermentation of sugars and glycerin. This is normally to be studied in fermentation tubes. Ordinarily use beef-extract broth containing 1 per cent of the substance investigated; but if the organism does not grow well in such broth and some medium is known in which it does grow well, the latter may be used. Generally speaking, organisms of series I and II should be studied in broth, organisms of series III and IV in some other medium. Incubate organisms of series I and III at 37°, organisms of series II and IV at 25°. Test ordinarily on 1st, 3rd, and 7th days, although the best days for testing will depend upon the rapidity of growth of the culture. Inoculations should always be made at least in triplicate.

To test for acid, it is recommended that in place of the illogical titrimetric method, determinations of hydrogen-ion concentration be made by the colorimetric method described by Clark and Lubs (1917a). In accurate research work the exact shade of the indicator should be compared with that obtained in standard "buffer" solutions, and results recorded in terms of P_H. In laboratories where these standard solutions cannot be obtained, it is better to record results simply as + or −, according to the reaction of the culture to litmus, than to use the titration method. Under such conditions it is possible, however, to obtain a rough idea of the hydrogen-ion concentration by the use of Clark and Lubs' series of indicators without making accurate determinations of P_H. Four different degrees of acidity can be easily distinguished by this simple method in sugar broth with initial reaction of neutrality. The indicator reactions for these different degrees of acidity are listed in table 1, together with the approximate range of P_H to which each corresponds. In the absence of accurate determinations, these degrees of acidity may be recorded by the indefinite terms, "weak," "moderate," "strong" and "very strong," or by the symbols +, ++, +++, and ++++.

Gas production is ordinarily measured in percentage of gas in the closed arm, and the ratio of H: CO_2 by means of absorption with NaOH, using the methods described in laboratory manuals (filling open arm with 4 per cent NaOH, allowing gas to enter open arm, shaking and returning gas to closed arm). As this method is far from accurate, it is recommended for provisional use only.

The fermentation test is ordinarily of no significance for organisms of series III and IV because of their poor growth in broth. Sometimes these organisms can be studied in some other liquid medium in which they do give good growth; but often it is necessary to use agar slants. In such a case, use a sugar agar containing brom cresol purple (see p. 117). Litmus can be used, but is unsatisfactory. Often gas-production can be detected in agar cultures by the presence of cracks and air bubbles but, as a test for gas, agar slants are not as reliable as fermentation tubes.

Milk. Acid production in milk can be detected by adding brom cresol purple to the culture and comparing with the color obtained by adding the same proportionate quantity of indica-

TABLE 1

Degrees of acidity easily recognized in clear media

ACIDITY	INDICATOR REACTIONS	APPROXIMATE PH-VALUE
"Neutral".....	Blue or green to brom thymol blue*	Over 6.2
"Weak".....	Yellow to brom thymol blue Purple to brom cresol purple*	5.2–6.0
"Moderate".....	Yellow to brom cresol purple Orange to methyl red†	4.6–5.0
"Strong".....	Maximum red to methyl red Blue or green to brom phenol blue*	3.2–4.4
"Very strong"....	Yellow to brom phenol blue	Under 3.0

* Use a 0.04 per cent alcoholic solution.
† Use a 0.02 per cent alcoholic solution.

TABLE 2

Degrees of acidity easily recognized in milk

ACIDITY	INDICATOR REACTION, ETC.	APPROXIMATE PH-VALUE
"Neutral".....	Same color with brom cresol purple* as sterile milk; i.e., blue to gray-green	6.2–6.8
"Weak".....	Color with brom cresol purple lighter than in sterile milk; i.e., gray-green to greenish yellow	5.2–6.0
"Moderate".....	Yellow with brom cresol purple. Not curdled	4.7–5.0
"Strong".....	Curdled. Blue or green to brom phenol blue*	3.2–4.6
"Very strong"....	Yellow to brom phenol blue	Under 3.0

* Use a 0.04 per cent alcoholic solution.

tor to sterile milk. (Brom thymol blue does not give satisfactory results in milk.) Four degrees of acidity that can be simply recognized in milk are listed in table 2. They correspond closely to those listed in table 1, differing only in that brom

cresol purple is used instead of brom thymol blue to show "neutrality" and that the curdling point (P_H =4.7) is used to separate between "moderate" and "strong" acidity instead of the less definite point of maximum red to methyl red. The same methods of expression used in recording acidity in clear media should be used in recording that of milk.

Litmus milk often gives valuable information, showing not only the production of acid, but also decolorization of the litmus by organisms that are able to reduce it. More accurate results as to acidity can be obtained by using brom cresol purple, as shown by Clark and Lubs (1917b). This indicator, however, does not show the reduction phenomena which are sometimes of diagnostic value in litmus milk cultures; so its substitution for litmus is not always to be recommended.

Reduction of nitrates. For routine work, nitrate broth should have the composition given on p. 116. Always use this broth first unless the organism is known to grow poorly in it. If an organism does not give good growth in this medium (as indicated by distinct cloudiness and precipitate), a negative nitrite test in this medium is meaningless. In such a case record the results of the test as doubtful, putting a question mark in the group-number at the point which indicates action on nitrates; or else make the test in some nitrate-containing, nitrite-free medium in which the organism does produce good growth. For instance, if (like *B. communis* and most pathogens) it requires considerable organic matter, use 2 or even 5 grams of peptone per litre; or if it grows poorly in the presence of organic matter, use some nitrite-free synthetic medium in which it is known to grow. The tubes should be inoculated in triplicate and incubated at 37° for organisms of series I and III; at 25° for organisms of series II and IV.

To test for nitrite the following reagents are necessary: (1) dissolve 8 grams sulphanilic acid in 1 litre of 5N acetic acid (1 part glacial acetic acid to 2.5 parts of water) or in 1 litre of dilute sulphuric acid (1 part concentrated acid to 20 parts water); (2) dissolve 5 grams α-naphthylamine in 1 litre of 5N acetic acid or of very dilute sulphuric acid (1 part concentrated acid

to 125 parts water). Add a few drops of each of these reagents to 3 to 5 cc. of the culture to be tested. A distinct pink or red indicates the presence of nitrite. Always test a sterile check that has been kept under the same conditions, to guard against errors due to the absorption of nitrite from the air.

Gas is rarely produced from nitrates, and when produced is generally to be observed as bubbles in an open test tube. If its presence is suspected, however, the culture should be tested in a fermentation tube.

In interpreting results, it must be remembered that the absence of nitrite in a culture showing poor growth does not indicate that the organism cannot reduce nitrates. If nitrate reduction is observed in any medium whatever, the organism is to be recorded as a nitrate-reducer; but unless the routine formula is used, the exact composition of the medium must always be given.

Chromogenesis. Color production should be recorded if observed in broth, on beef-extract agar, gelatin or potato, or if noticed to a striking extent on any other medium. In the group number, the point devoted to chromogenesis refers to the color produced on beef-extract agar.

Diastatic action on starch. Provisional method. Use beef-extract agar containing 0.2 per cent of soluble starch. Pour into a petri dish, and after hardening make a streak inoculation on its surface. Incubate at 37° for organisms of series I and III, at 25° for organisms of series II and IV. Determinations for the group number shall be based upon results obtained on the seventh day. To make the test, flood the surface of the petri dishes with a saturated solution of iodine in 50 per cent alcohol. The breadth of the clear zone outside of the area of growth indicates the extent of diastatic action. If over 2 mm. in width on the seventh day it shall be recorded as "strong;" if under 2 mm. as "feeble;" if no clear zone is present, as "absent."

This method is advised by P. W. Allen (1918) and, although it is the best method yet called to the attention of the committee, it is recommended only for provisional use. It requires some

modification e.g., reducing the amount of peptone in the medium, for organisms that grow so rapidly as to cover the entire surface of the plate in seven days, thus leaving no room for a clear zone outside.

The group-number is a brief means of recording the salient features of the organism. It is primarily a summary of the physiological characteristics just discussed. As each of the determinations is made, the proper figure for that place in the group number is to be checked or underscored. After completing the determinations, the entire group number is to be written in at the place left for it on the chart. The genus symbol should precede the group number. The present group number, adopted by the Society in 1907, is intended for use with the generic names of Migula. As Migula's genera are not in such general use today as they were ten years ago, a revision of the group number on some other basis will be necessary in the near future.

Brief characterization. On the right hand margin of page one of the chart is a place for recording by a + or − sign other important characteristics of the organism (primarily cultural) not included in the group number. This margin together with the group number constitute a brief characterization of the organism,—a summary of the tests outlined above.

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GLOSSARY OF TERMS USED ON THE CHART

Adherent, Applied to sporangium wall, indicates that remnants of sporangium remain attached to endospore for some time.

Aerobic, growing in the presence of free oxygen; **strictly aerobic,** growing *only* in the presence of free oxygen.

Amorphous, without visible differentiation in structure.

Anaerobic, growing in the absence of free oxygen; **strictly anaerobic,** growing *only* in the presence of free oxygen; **facultative anaerobic,** growing both in presence and in absence of free oxygen.

Arborescent, branched, tree-like growth.

Beaded, in stab or stroke culture, disjointed or semi-confluent colonies along the line of inoculation.

Bipolar, at both poles or ends of the bacterial cell.

Brittle, growth dry, friable under the platinum needle.

Butyrous, growth of butter-like consistency.

Chains, four or more bacterial cells attached end to end.

Chromogenesis, the production of color.

Ciliate, having fine, hair-like extensions, resembling cilia, sometimes not visible to the naked eye.

Clavate, club-shaped.

Coagulation, the separation of casein from whey in milk.

Contoured, an irregular, smoothly undulating surface, like that of a relief map.

Convex, surface the segment of a sphere.

Crateriform, a saucer-shaped liquefaction of the medium.

Cuneate, wedge-shaped.

Curled, composed of parallel chains in wavy strands, as in anthrax colonies.

Diastatic action, conversion of starch into simpler carbohydrates, such as dextrins or sugars, by means of diastase.

Echinulate, a growth along line of inoculation with toothed or pointed margins.

Effuse, growth thin, veily, unusually spreading.

Endospores, thick-walled spores formed within the bacterial cell; i.e., typical bacterial spores like those of *B. anthracis* or *B. subtilis.*

Entire, with an even margin.

Erose, border irregularly toothed.

Filaments, applied to morphology of bacteria, refers to thread-like forms, generally unsegmented; if segmented, to be distinguished from chains (q. v.) by the absence of constrictions between the segments.

Filamentous, growth composed of long, irregularly placed or interwoven threads.

Filiform, in stroke or stab cultures, a uniform growth along line of inoculation.

Flocculent, containing small adherent masses of bacteria of various shapes floating in the culture fluid.

Fluorescent, having one color by transmitted light and another by reflected light.

Granular, composed of small granules.

Infundibuliform, form of a funnel or inverted cone.

Iridescent, exhibiting changing rainbow colors in reflected light.

Lobate, having the margin deeply undulate, producing lobes (see *undulate*).

Luminous, glowing in the dark, phosphorescent.

Maximum temperature, temperature above which growth does not take place.
Membranous, growth thin, coherent, like a membrane.
Minimum temperature, temperature below which growth does not take place.
Mycelioid, colonies having the radiately filamentous appearance of mold colonies.
Napiform, liquefaction in form of a turnip.
Opalescent, resembling the color of an opal.
Optimum temperature, temperature at which growth is most rapid.
Papillate, growth beset with small nipple-like processes.
Pellicle, bacterial growth forming either a continuous or an interrupted sheet over the culture fluid.
Peptonization, rendering curdled milk soluble by the action of trypsin.
Peritrichiate, covered with flagella over the entire surface.
Persistent, lasting many weeks or months.
Plumose, a fleecy or feathery growth.
Polar, at the end or pole of the bacterial cell.
Pulvinate, decidedly convex, in the form of a cushion.
Punctiform, very small, but visible to naked eye; under 1 mm. in diameter.
Radiate, showing ray-structure.
Raised, growth thick, with abrupt or terraced edges.
Reduction, removing oxygen from a chemical compound. Refers to the conversion of nitrate to nitrite, ammonia, or free nitrogen, and to the decolorization of litmus.
Rhizoid, growth of an irregular branched or root-like character, as in *B. mycoides.*
Ring, growth at the upper margin of a liquid culture, adhering to the glass.
Rapid, developing in 24 to 48 hours.
Rugose, wrinkled.
Saccate, liquefaction in form of an elongated sac, tubular, cylindrical.
Slow, requiring 5 or 6 days for development.
Spindled, larger at the middle than at the ends. Applied to sporangia, refers to the forms frequently called *clostridia.*
Sporangia, cells containing endospores.
Spreading, growth extending much beyond the line of inoculation, i.e., several millimeters or more.
Stratiform, liquefying to the walls of the tube at the top and then proceeding downwards horizontally.
Transient, lasting a few days.
Truncate, ends abrupt, square.
Turbid, cloudy with flocculent particles; i.e., cloudy plus flocculence.
Umbonate, having a button-like, raised center.
Undulate, border wavy, with shallow sinuses.
Viscid, growth follows the needle when touched and withdrawn; sediment on shaking rises as a coherent swirl.

THE ENZYMES OF THE TUBERCLE BACILLUS

H. J. CORPER AND H. C. SWEANY

From the Laboratories of the Municipal Tuberculosis Sanitarium of the City of Chicago

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The tubercle bacillus and the related acid fast bacilli (lepra, smegma, etc.,) have for a great many years been looked upon as distinct and different organisms, biologically and chemically, from the ordinary rapidly growing bacteria, and it is rather singular that until recently the tubercle bacillus should have been considered not to possess the ordinary well-known enzymes. This lack of knowledge as to the presence of enzymes in the tubercle bacillus is, of course, explicable on the basis that not until within recent years have sufficiently delicate quantitative methods been available for the demonstration of these interesting catalytic substances in such a sluggish organism as the tubercle bacillus and in amounts so small or of so slight an activity as those characteristic of this organism. It is worthy of note also that these problems could not have been solved before the days of the use of antiseptics in studying enzymatic action. During the course of investigations carried out to find a suitable antigen for use in complement fixation tests for tuberculosis, it was deemed advisable to understand more fully the part played by the enzymes in the tubercle bacillus and for this purpose the experiments to be reported upon were carried out.

LITERATURE

In order to study the action of the enzymes of various bacteria upon different substances and thus gain a better insight into the nature of these enzymes, Eijkman (1901) used a simple plate method called the "auxanographic" method of Beijerinck or the diffusion method of Wijsman. This method, which consists in mixing various substrates (casein, blood, starch, tallow,

cellulose, etc.,) with nutrient agar and observing the effect of the growths of various organisms upon the substrates, was of necessity crude and gave only gross results. The criterion of enzymatic action was merely the formation of a zone of clearing or change in the media surrounding the colonies. Thus Eijkman found that the following gelatin liquefying or digesting organisms also cause the solution of casein: *B. anthracis, B. pyocyaneus, Staphylococcus pyogenes aureus, B. Metschnikowi, B. cholerae, B. fluorescens, B. prodigiosus, B. indicus, B. ruber, B. subtilis, B. megatherium,* and *B. mesentericus;* while the following organisms, not peptonizing gelatin, also failed to dissolve casein: *B. typhosus, B. coli-communis, B. mallei, B. pestis, B. diphtheriae* and *B. lactis cyanogenes.* He concludes, therefore, that the same enzyme probably digests both substances.

As the hemolytic action of a number of bacteria (*B. diphtheriae B. anthracis, B. fluorescens, B. mesentericus, B. prodigiosus* and *B. indicus*) did not vary hand in hand with the gelatin liquefying property, he concludes that the hemolytic and tryptic enzymes are not identical, but suggests that different tryptic enzymes may be present in different bacteria and these might in some cases attack blood or gelatin more vigorously. Varying grades of diastatic action by pathogenic organisms were observed with starch. *B. anthracis* and *B. cholerae* intensely digested it, while *B. diphtheriae* and *B. dysenteriae* (Kruse) had only a faint action and *B. mallei, B. pyocyaneus* and *Staphylococcus pyogenes aureus* were negative in this respect. Fat splitting power was also found to be present in some organisms and absent in others. None of those forms tested were able to split beeswax. In all tests with inulin, (Eijkman, 1913) keratin and cellulose negative results were obtained. Elastic tissue prepared from calf lung was digested by *B. pyocyaneus* (culture and bouillon filtrate),—which action was destroyed at 80°C.,—*B. anthracoides* and a bacillus isolated from a case of lung gangrene. It was noteworthy that all the elastic tissue dissolving organisms liquefied gelatin, but the reverse did not hold true.

Fontes (1911) was unable to demonstrate enzymes, zymases or oxidases in cultures or tuberculins of bovine and human tubercle

bacilli. Proteolytic enzymes were also not found in tuberculous pus free from other organisms. Opie and Barker (1908) had earlier observed that tuberculous tissues in the early stages contained a proteolytic enzyme acting in neutral and weakly acid solution. With the advance of caseation the one acting in alkaline solution disappears while the other (lympho-protease) is retained. After complete caseation the latter also disappears. No enzymes were demonstrable in tuberculous exudates in human beings (Opie and Barker, 1909).

Gosio (1905) using the tellurite and selenite reduction test devised by him was able to demonstrate reductases in all the bacteria tested including the various tubercle bacilli: avian, bovine and human. He examined 173 microorganisms and divided them into three classes, dependent upon the reaction obtained: (1) a decided reaction, (2) a less intense, but fully evident reaction (in this class he included a bovine, a human and an avian tubercle bacillus and a so-called pseudo-tubercle-bacillus (Rabinowitsch)), (3) a very slight reaction.

Wells and Corper (1912) studying the lipase of *B. tuberculosis* and other bacteria noted that toluene would kill the tubercle bacillus but did not destroy the lipases, and that lipolytic enzymes are present with different activites in the organisms tested (*B. dysenteriae, Staphylococcus pyogenes aureus, B. pyocyaneus, B. coli* and *B. tuberculosis*). Of these the tubercle bacillus was least actively lipolytic. They also noted that the "auxanographic" method was not suitable for use in studying the enzymes of the tubercle bacillus.

Kendall, Day and Walker (1914c) verified and elaborated these findings noting that various strains of the human tubercle bacillus, the bovine and avian bacilli as well as the leprosy, smegma and grass bacilli, form lipase during their growth on glycerin broth. This lipase is present in the medium free from bacteria. The bodies (Kendall, Day and Walker, 1914d) of acid fast bacteria grown in nutrient broth, with glucose, mannite and glycerin as additional sources of carbon, freed from adherent media, also contained a lipase not as active or as great in amount as that in the culture media. The authors were

unable to determine whether the lipase was freed as the result of autolysis or whether it was excreted as an exo-lipase.

The above authors (Kendall, Day and Walker, 1914d) also observed that the metabolism of the smegma and grass bacillus resembles that of the rapidly growing human tubercle bacillus in two important particulars; neither glucose, mannite nor glycerin exhibits any sparing action for the protein constituents as measured by the ammonia content of the broth (the "lepra bacillus" does not present this metabolic phenomenon); and a rapidly growing strain of human tubercle bacilli grown in a medium of very simple composition consisting essentially of diammonium hydrogen-phosphat, as a combined source of nitrogen and phosphorus, and glucose, mannite and glycerin respectively, as a source of carbon, so changed this medium that 10 per cent of the ammonium nitrogen had disappeared at the end of two weeks apparently being built up into bacillary bodies. At the end of four weeks between 40 and 50 per cent of the lost ammonia nitrogen reappeared in the media. This occurred coincidently with the cessation of vegetative activity, strongly suggesting, as stated by the authors, that it is associated with a certain amount of autolysis of the bacilli, (Kendall, Day and Walker, 1914a).

That the tubercle bacilli themselves contain enzyme inhibiting substances was shown by Jobling and Peterson (1914) who prepared unsaturated fatty acid soaps from tubercle bacilli which were more actively inhibitory than soaps prepared from linseed, olive and cod liver oils.

In the following investigations it will be observed that an attempt was made to check the findings obtained in every case by as many methods as possible. Frequent failures marked the progress of the work however, because certain of the methods used were not delicate enough for the purpose and others could not be used on account of interfering (absorption and turbidity) phenomenon. This was especially true of the nephelometric methods tried.

AUTOLYTIC ENZYMES

Method

The amount of non-coagulable nitrogen was determined in all the following experiments, except where otherwise indicated, by the colorimetric micro-method as devised by Folin and Farmer (1912) using the distillation modification of Bock and Benedict, (1915). The coagulable nitrogen was removed by the addition of ten volumes of 2.5 per cent trichloracetic acid according to Greenwald (1915). The results obtained were plotted in curves, using the initial nitrogen found as the zero point, that is the amount of non-coagulable nitrogen obtained in a definite volume (1 cc. of solution) at the beginning of the experiment was subtracted from the amount of nitrogen in an equivalent amount (1 cc.) of solution subsequently.

The method used for determining quantitatively the amount of amino acid α nitrogen was that devised by Harding and MacLean (1915; 1916). Asparagin was used as a standard and since the results are merely comparative the objections to the use of asparagin raised by the above authors did not apply to the procedure.

EXPERIMENTAL PART

Series I. Liberation of nitrogenous substances by tubercle bacilli suspended in salt solution

In order to determine whether there was a liberation of non-coagulable nitrogenous substances from tubercle bacilli under certain conditions, heavy suspensions were made of virulent human tubercle bacilli in sterile physiological salt solution and divided among three graduated centrifuge tubes in as nearly equal amounts as possible (each tube containing 10 cc. of heavy suspension). At the conclusion of the experiment the amount of residual nitrogen remaining in the bacilli was always determined and found to agree fairly well within the limits of error of the method, thus serving as a check upon the use of approxi-

mately equivalent amounts of bacilli in the three tubes. One of these tubes was heated as a control and kept in the incubator, one was placed in the incubator at 37°C., without previous heating and the third was kept at room temperature during the course of the experiment in order to determine the most favorable condition suitable for the liberation of the nitrogenous substances from the bacilli.[1]

It was found that tubercle bacilli suspended in physiological salt solution in this way liberate non-coagulable nitrogenous substances at incubator temperature (37°C.), but that this liberation requires a number of days. At room temperature (i.e., about 15° to 20°C.) this does not occur to any appreciable extent within the same time.

Series II. Determination of an autolysis of human tubercle bacilli

In order to determine whether the non-coagulable nitrogenous substances liberated at incubator temperature are merely dissolved out from the bacillary bodies of these substances or whether they are formed as a result of enzymatic action as suggested by Kendall, Day and Walker, tubercle bacilli in equal amounts in two tubes were suspended in sterile physiological salt solution and one was incubated at 37°C., as a control, while to the other was added 3 cc. toluene before incubation. The toluene, as has been shown by Wells and Corper (1912), kills the bacilli but leaves the enzymes intact.[2]

It appeared that there was a definite liberation of non-coagulable nitrogen in both tubes, rather more rapid and reaching a maximum earlier under toluene than in sterile physiological salt solution. A suggested explanation of the difference may be found in the fact that the bacilli are killed rapidly by the toluene and therefore more rapidly disintegrate as a result of enzymatic action, an autolysis.

[1] Charts depicting these results and those of Series II have been published in the Jour. of Infectious Diseases, Vol. 19, 1916, p. 315–21.

[2] See note under series I.

Series III. Determination of an autolysis of bovine tubercle bacilli

The experiments of series I and II were performed with human tubercle bacilli; this series is in a certain sense a repetition and check on the above except for the fact that bovine tubercle bacilli were used. These results are plotted in chart I.

They bear out the findings with the human tubercle bacilli in that the autolysis occurs more rapidly under toluene (antiseptic) than in merely aseptic conditions.

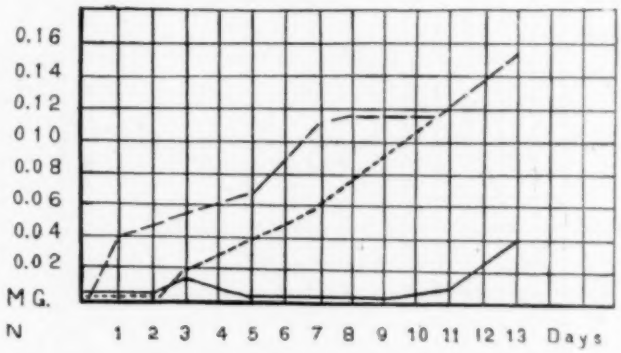

CHART I. AUTOLYSIS OF TUBERCLE BACILLI NON-COAGULABLE NITROGEN CURVES

Continuous line is heated control at incubator temperature. Dotted line is aseptic autolysis at incubator temperature. Dash line is antiseptic (toluene) autolysis at incubator temperature.

Series IV. Liberation of amino acids during autolysis of tubercle bacilli

As a check upon the above findings and in order to see whether the increase in non-coagulable nitrogen runs parallel with an increase in liberated amino acid α nitrogen, the above experiments were repeated and the autolysate was tested, at various intervals during incubation, for α amino acid content by the

Harding and MacLean colorimetric method. The results are plotted in chart II.

It is to be noted again that even though equivalent amounts of human tubercle bacilli were used in each test the curve for the antiseptic (toluene) autolysis is higher than the aseptic curve and that autolysis as indicated by the liberation of amino acids is more rapid at incubator (37°C.) than at room tempera-

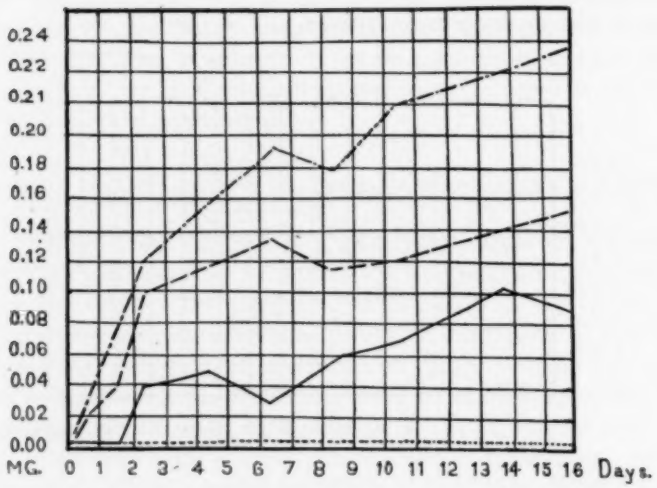

CHART II. AUTOLYSIS OF TUBERCLE BACILLI. AMINO ACID CURVES

Solid line is aseptic autolysis at room temperature. Dotted line is heated control. Long dash line is aseptic autolysis at incubator temperature. Dash dot line is antiseptic (toluene) autolysis at incubator temperature.

ture (these experiments were carried out in a warmer room than those reported in series I which probably accounts for the greater height attained by the room temperature curve).

Series V. Liberation of antigenic substances during autolysis

In order to determine whether, coincident with the autolysis of tubercle bacilli occurring at incubator temperature, there was

also an increase in soluble tuberculosis antigen in the autolysate available for complement fixation tests, that is capable of uniting with tuberculosis antibody, human and bovine tubercle bacilli were suspended in sterile physiological salt solution and kept in the incubator at 37°C. At definite intervals the clear autolysate was tested for non-coagulable nitrogen content and titrated for its antigenic strength. The antigenic titre was obtained by determining the minimum amount necessary to give a complete binding of all the complement when using a definite amount of + + + + antituberculosis (human) serum. The Noguchi antihuman hemolytic system was used in these tests. The results are tabulated in Chart III.

	Nitrogen Curves	Antigen Curves							
Days	M G.N. PER C C	0.2 C C	0.1 C C	0.5 C C	0.01 C C	0.005 C C	0.001 C C	0.0005 C C	0.0001 C C
0	0.00	++++	++++	+++	−	−	−	−	−
1	0.00	++++	++++	+++	±	−	−	−	−
2	0.01	++++	++++	++++	+++	±	−	−	−
3	0.02	++++	++++	++++	++++	+++	+ +	+	−
4	0.03	++++	++++	++++	++++	++++	+++	+	±
6	0.05	++++	++++	++++	++++	++++	++++	+ +	+ +
8	0.06	++++	++++	++++	++++	++++	++++	+ + +	+ +
10	0.08	++++	++++	++++	++++	++++	++++	+ + +	+
13	0.15	++++	++++	++++	++++	++++	++++	+ + +	+ +

CHART III. CORRELATION OF AUTOLYSIS AND ANTIGEN FORMATION

It is to be noted that coincident, although not absolutely parallel, with the increase in non-coagulable nitrogen liberated there also occurs an increase in the antigenic titre of the autolysate, so that an autolysate which only gave a titre of 0.1 cc. on the first day gradually and consistently increases to a titre of 0.001 cc. on the sixth day.

DETERMINATION OF INDIVIDUAL ENZYMES OF THE TUBERCLE BACILLUS

In the hope of being able to determine the presence of the various individual enzymes in the tubercle bacillus by the nephelometric method for determining enzymatic action recently

described by Kober and his colleagues (Graves and Kober, 1914; Kober, 1913; Kober and Graves, 1914), a series of experiments were carried out in which heavy emulsions of tubercle bacilli were mixed with definite amounts of various substrates (sodium caseinate for trypsin, acid casein for erepsin, acid edestin for pepsin, nucleic acid for nucleases, starch for diastase, sucrose for invertase, and urea for urease). In the majority of cases this method was found impractical, however, on account of the adsorption of the protein substrates by the tubercle bacilli, which made quantitative determinations impossible. In a number of instances this difficulty was, however, overcome by determining the presence of the enzymes in the autolysate where no adsorption occurs and checking their presence by other methods.

Methods

The methods used for quantitative nephelometric determination of the various substrates (casein, edestin and nucleic acid) were those described by Kober and his colleagues, the previously cited micro-method of Folin, modified by Bock and Benedict, for determining non-coagulable nitrogen, and the amino acid α nitrogen method of Harding and MacLean. The presence of urease was determined by the aeration method of Folin (Folin and MacCollum, 1912) and the presence of diastase and invertase by the amount of glucose liberated from starch and sucrose by the recent picramic acid method of Lewis and Benedict (1915) as used by Myers (1916) and Myers and Rose, (1916), for determining the presence of these enzymes in ptyalin and the presence of glucose, sucrose, dextrin and starch in foodstuffs. The quantitative Fehling method proved far too inaccurate and not delicate enough for this purpose.

Series I. Proteolytic enzyme acting in alkaline solution (trypsin-like enzyme)

Experiment 1. By its presence in the autolysate. (a) In a graduated 15 cc. centrifuge tube was emulsified 2 cc. of human tubercle bacilli and sufficient sterile physiological salt solution

was added to make a volume of 12 cc., and, as antiseptic, 2 cc. toluene and 1 cc. chloroform. After thoroughly mixing, this was placed in the incubator at 37°C. for twenty-four hours, centrifugated at the end of that time and the supernatant autolysate withdrawn, filtered through a hard filter paper and divided into two equal parts; one part heated for thirty minutes at 100°C., as control to destroy the enzymes, and the other half kept intact. To each was added 1 cc. 0.1 per cent sodium caseinate, sodium carbonate to make 0.3 per cent, 1 cc. toluene and 1 cc. chloroform. The tubes containing these mixtures were then incubated at 37°C., and a definite amount of the clear watery solution withdrawn at various intervals and compared, control and test, nephelometrically after precipitation with sulphosalicylic acid. The results obtained, figured in percentage of the heated control, were as follows:

	0	2 DAYS	4 DAYS	6 DAYS	12 DAYS
Percentage of original casein solution added...	100	82.2	79.6	78	63

The above figures indicate a definite splitting of the casein by the autolysate withdrawn at one day.

(b) The above experiment 1a was repeated but the autolysate was withdrawn after twelve days in the incubator at 37°C. The results obtained with the autolysate as compared to the heated control were:

	0	3 DAYS	6 DAYS	9 DAYS	12 DAYS
Percentage of original casein solution added...	100	80	74.4	73	71.4

These figures indicate a definite hydrolysis of the casein by the autolysate withdrawn at twelve days.

(c) In order to check this point more fully a more complete experiment was performed. Human tubercle bacilli, 2 cc. by volume, were suspended in physiological salt solution to make a total volume of 12 cc., and 2 cc. toluene and 1 cc. chloroform added. The mixture was incubated for three days, centrifu-

gated and about 10 cc. of autolysate withdrawn. This was then replaced by physiological salt solution and the entire tube incubated another three days. The same process was again repeated at nine and twelve days. The autolysate after the various intervals of three, six, nine and twelve days, was divided into two equal portions (1 portion to be heated for control and the other to be retained intact). To each of these was added

TABLE 1

Period of determination after adding substrate

AGE OF AUTOLYSATE		THREE DAYS	SIX DAYS	NINE DAYS
		per cent	*per cent*	*per cent*
Three days	T*	109	94	68
	H	125	125	121
Six days	T	83	87	84
	H	97	106	107
Nine days	T	89	83	87
	H	97	100	103
Twelve days	T	91	86	87
	H	98	97	98

* T designating the test for enzyme and being the percentage of casein recovered as co npared with a standard containing the amount of casein originally added; H, designating the percentage of casein in the heated control as compared with the same standard containing the amount of casein originally added. The data are given in this form to rule out any conflicting turbidity or sources of error which may have been occasioned by the autolysate itself.

When these experiments were performed it was not realized that there were any conflicting turbidities formed or other sources of error and so a test sample was not withdrawn immediately, it being taken for granted that the heated control would suffice for this purpose.

1 cc. 0.1 per cent sodium caseinate, enough sodium carbonate to make 0.3 per cent, 1 cc. toluene and 1 cc. chloroform. This mixture was incubated at 37°C., and a definite amount withdrawn after definite intervals of incubation (three, six and nine days) and after precipitation with sulphosalicylic acid tested nepholometrically for casein content. The results are given in table 1.

These results indicate a definite splitting of the casein by the autolysate withdrawn at the third day of autolysis, but subsequently the enzymes have either been entirely washed away from the bacilli or have been so diluted by the additional salt solution added that they are incapable of exerting any action upon the casein.

Experiment II. By the non-coagulable nitrogen liberated in alkaline solution. Tubercle bacilli, 4 cc. of a heavy emulsion, were placed in 15 cc. graduated centrifuge tubes (2 cc. in each). One as control was heated to kill the bacilli, and to both was added 10 cc. physiological salt solution, sufficient sodium carbonate to make 0.3 per cent, 2 cc. toluene and 1 cc. chloroform. The tubes were then placed in the incubator at 37°C., after withdrawing 1 cc. as a control test, and 1 cc. of the clear supernatant solution was withdrawn after centrifugation daily, for eight days. The non-coagulable nitrogen was determined as before by the Folin micro-method.

	DAYS									RESIDUAL NITROGEN
	0	1	2	3	4	5	6	7	8	
										mgm.
Test	0.12	0.12	0.13	0.13	0.14	0.14	0.15	0.16	0.17	4.26
Heated control	0.11	0.11	0.11	0.10	0.11	0.12	0.12	0.11	0.12	4.8

The figures are given in milligrams of nitrogen per cubic centimeter

These figures indicate that there is present in tubercle bacilli an enzyme capable of decomposing the nitrogenous constituents of the bacillary bodies in alkaline solution.

Experiment III. By the amino acid α nitrogen liberated in alkaline solution. The above experiment (II) was repeated in all details except that the solution was freed from coagulable proteins by means of fifteen volumes of methyl alcohol, the alcohol evaporated off from the filtrate, the residue taken up by 2 cc. distilled water and this tested quantitatively for amino acid α nitrogen content. The following results were obtained, the figures being given in milligrams amino acids using asparagin as the standard.

	DAYS							
	0	1	2	3	5	7	10	30
Test	0.05	0.06	0.06	0.07	0.08	0.09	0.12	0.14
Heated control	0.06	0.06	0.07	0.05	0.05	0.05	0.06	0.05

This experiment indicates that there is an enzyme in tubercle bacilli capable of splitting off into simple form the amino acid building stones of the bacillary bodies.

Summary. The evidence obtained as to the action of the enzymes of the tubercle bacilli (autolysate) upon casein in alkaline solution and upon the bacillary bodies themselves in alkaline solution seem to indicate that this micro-organism possesses a proteolytic enzyme resembling trypsin in action.

Series II. Proteolytic enzyme acting in acid solution (pepsin-like enzyme)

Experiment I. By its presence in the autolysate. The nephelometric method in which edestin was used was not found to be serviceable for this purpose since physiological salt solution partially precipitated the edestin, and if distilled water was used as a solvent and for preparing the autolysate substances producing a turbidity were obtained from the tubercle bacilli.

Experiment II. By the non-coagulable nitrogen liberated in acid solution. The differences obtained in the non-coagulable nitrogen figures by the Folin micro-method, colorimetrically, were not sufficient to warrant drawing any conclusions.

Experiment III. By the amino acids liberated in acid solution. When using suspensions of bacilli (2 cc. bacillary sediment), killed by toluene (2 cc.) and chloroform (1 cc.) the solution being made 0.2 per cent acid with hydrochloric acid and tested at definite intervals as in series I, experiment III, for amino acid α nitrogen liberated, the results obtained were as follows:

	DAYS							
	0	1	2	3	5	7	10	30
Test	0.04	0.05	0.07	0.07	0.07	0.08	0.08	0.08
Heated control	0.05	0.06	0.05	0.06	0.05		0.05	0.04

Summary. Tubercle bacilli possess a pepsin-like enzyme which, though feeble in action, is capable of liberating amino acid α nitrogen from the bacillary bodies in the presence of 0.2 per cent hydrochloric acid.

Series III. Erepsin-like enzymes

Attempts were made to determine the presence of erepsin-like enzymes by using casein in acid solution and testing nephelometrically, as recommended by Kober, by using both the bacillary emulsion and autolysate therefrom, but both attempts failed on account of turbidities produced in the solutions by the bacillary emulsion and autolysate even before adding the precipitant. It was finally decided to test for erepsin-like enzymes by preparing a peptone from Witte's peptone which was completely precipitated by ten volumes of methyl alcohol so that no perceptible trace was dissolved therefrom by means of methyl alcohol, and using as criterion the splitting of this peptone in acid solution, testing for such splitting by means of Harding and MacLean's quantitative amino acid α nitrogen test.

Four cubic centimeters of tubercle bacilli were diluted with sterile physiological salt solution to 10 cc., 2 cc. toluene and 1 cc. chloroform were added, and the mixture, after shaking thoroughly, was placed in the incubator at 37°C. for twenty-four hours, after which the centrifugated supernatant liquid was filtered through a sterile hard filter paper and divided into two equal portions. One portion (5 cc.) was heated thirty minutes at 100°C., while the other portion (5 cc.) remained unheated. To each was added sufficient sterile physiological salt solution to make 10 cc., sufficient hydrochloric acid to make 0.2 per cent, 2 cc. toluene, 1 cc. chloroform and 1 cc. 0.25 per cent pure peptone solution (completely precipitable by ten volumes of methyl alcohol). From each of these tubes a control of 0.5 cc. was withdrawn and to this ten volumes of methyl alcohol was added, the whole centrifugated at 3000 revolutions until perfectly clear and the supernatant clear methyl alcohol analyzed quantitatively for amino acid content. At definite intervals

after incubation 0.5 cc. amounts were withdrawn and also analyzed for methyl alcohol soluble amino acid α nitrogen content. In making the analyses the amount of amino acids was always compared with a fresh solution of asparagin as standard (0.0317 mgm. amino acid α nitrogen as asparagin) and the figures given are in milligram amino acid α nitrogen as derived from this standard.

	DAYS						
	0	1	2	3	5	7	10
Erepsin test	0.015	0.023	0.024	0.024	0.026	0.026	0.032
Heated control	0.019	0.023	0.022	0.022	0.022	0.019	0.021

Summary. The autolysate from tubercle bacilli possesses an enzyme capable, in 0.2 per cent hydrochloric acid solution, of splitting a completely methyl alcohol precipitable peptone prepared from Witte's peptone into simple amino acid α nitrogen compounds, not precipitable by this means.

Series IV. Nuclease (nucleic acid splitting enzyme)

Experiment I. Nephelometrically. To the autolysate (twenty-four hours in incubator under toluene) obtained from a heavy suspension of tubercle bacilli (2 cc. bacillary residue) in sterile physiological salt solution, was added (to a test and heated control) 1 cc. 0.1 per cent nucleic acid making a total of 6 cc., and 2 cc. toluene and 1 cc. chloroform. The nucleic acid was determined quantitatively by the nephelometric method of Kober. In this preliminary test the heated control was used as the standard and the results obtained in percentage as compared to heated control, were; immediately—100 per cent nucleic acid; three days—80 per cent nucleic acid; and six days 77.5 per cent nucleic acid.

This experiment was then repeated more elaborately in that both the heated control and test were compared with a freshly prepared standard of nucleic acid each time. The standard

contained 0.05 cc. of 0.1 per cent yeast nucleic acid in distilled water and was considered as 100 per cent. The total volume in this experiment was 10 cc. instead of 6 cc. as above and to this was added 1 cc. 0.1 per cent freshly prepared yeast nucleic acid solution. The results obtained, in percentage of the standard nucleic acid solution, were as follows:

	DAYS						
	0	1	3	4	5	7	10
Test	74.3*	56.6	56.0	52.8	51.3	49.1	50.5
Heated control	60.3†	59.8	60.0	60.2	60.6	59.7	58.4

* The turbidity produced in the test solution was greater than that accounted for by the nucleic acid added. No explanation has been found for this except the fact that certain substances may have been liberated by the autolysis of the tubercle bacilli which combine with the precipitant to form a turbidity.

† The turbidity of the heated control after the albumin precipitant was added was also greater than that accounted for by the nucleic acid added but less than that of the unheated test.

Summary. The autolysate from tubercle bacilli possesses an enzyme capable of splitting nucleic acid into simpler form.

Experiment II. In an attempt to corroborate the findings of experiment I by amino acid determinations, the following experiment was performed and the results are given for what they may be worth.

Two cubic centimeter amounts of bacillary residue were prepared in four tubes. Sufficient hydrochloric acid was added in one tube to make the final strength 0.1 per cent; to the second tube sufficient hydrochloric acid to make the final strength 0.1 per cent and 3 cc. 0.1 per cent acid caseinate; the third was kept as a neutral control; and to the fourth was added 3 cc. 0.1 per cent nucleic acid. All were diluted to 10 cc. with physiological salt solution, 2 cc. toluene and 1 cc. chloroform was added and the tubes incubated at 37°C. At various intervals definite amounts of the solution were withdrawn and tested for amino acid α nitrogen content.

	INTERVALS					
	0	36 HOURS	4 DAYS	6 DAYS	8 DAYS	11 DAYS
	mgm.	mgm.	mgm.	mgm.	mgm.	mgm.
1. HCl to 0.1 per cent	0.020	0.026	0.027	0.031	0.039	0.042
2. HCl to 0.1 per cent and 0.1 per cent acid caseinate	0.022	0.039	0.043	0.048	0.051	0.058
3. Neutral control	0.018	0.027	0.036	0.046	0.053	0.065
4. 3 cc. 0.1 per cent nucleic acid	0.024	0.025	0.028	0.034	0.041	0.043

Series V. Urease

The presence of urease in tubercle bacilli was determined by making an emulsion of human bacilli in physiological salt solution, 3 cc. bacillary residue made up to 32 cc. in each of two tubes, and adding 50 mgm. of urea and 5 cc. toluene to a heated control and a test. After withdrawal of a 3 cc. control (filtering through hard filter paper and using 2 cc. for analysis) the tubes were incubated at 37°C., and samples taken out for analyses at various intervals. The amount of urea decomposed was determined by the Folin aeration method. The enzyme action was stopped by the addition of 1 cc. saturated sodium carbonate to 2 cc. of the filtrate. The results obtained are expressed as milligrams of nitrogen in 2 cc. of filtrate.

	PERIOD OF WITHDRAWAL OF SOLUTIONS					
	0	18 HOURS	2 DAYS	3 DAYS	5 DAYS	8 DAYS
Urease test	0.020	0.060	0.100	0.135	0.134	0.132
Heated control	0.015	0.013	0.014	0.016	0.010	0.012

Summary. Tubercle bacilli possess an enzyme capable of decomposing urea.

Series VI. Diastase and Invertase

The presence of diastase and invertase in the tubercle bacillus was tested by determining the amount of reducing sugar formed from starch and sucrose as performed by Meyers and Rose

using the Lewis and Benedict colorimetric picramic acid method. Among four tubes was divided equally 14 cc. of bacillary emulsion (2 cc. bacillary residue in each). Two of the tubes were heated for thirty minutes at 100°C., in a water bath to destroy the enzymes present. To each of the four tubes was added 2 cc. toluene and 1 cc. chloroform and sufficient sterile physiological salt solution to make a total volume of 10 cc. of emulsion. To two of the tubes, one heated and one unheated, was added 5 cc. of 2 per cent sucrose making a concentration of 1 per cent sucrose, and to the other two, one heated and one unheated, 5 cc. of freshly prepared arrowroot starch emulsion made by adding 2 grams of starch in suspension in cold water to about 75 cc. boiling water and diluting to 100 cc. after cooling.

After thorough mixing, the tubes were centrifugated and 1 cc. of the solution drawn off and tested for reducing sugar. The tubes were then incubated at 37°C., and 1 cc. withdrawn and tested at various intervals thereafter.[3] The results are given in the following table.

		TIME OF TEST IN DAYS					
		0	1	3	6	8	10
Starch....	Not heated	Trace (about 0.1 mgm.)	0.2	0.2	0.2	0.2	0.2
	Heated control	Trace (about 0.15 mgm.)	0.15	0.15	0.2	0.2	0.2
Sucrose...	Not heated	Trace (about 0.15 mgm.)	0.15	0.25	0.3	0.3	0.3
	Heated control	Trace (about 0.2 mgm.)	0.2	0.2	0.25	0.25	0.25

Summary. Tubercle bacilli do not possess an enzyme capable of hydrolyzing starch or sucrose in sufficient amount to be demonstrable by the method used.

[3] Control tests of 1 per cent starch and 1 per cent sucrose to which had been added chloroform and toluene revealed no difference from a blank test with the reagents alone. Varying amounts of glucose, 0.5, 0.4, 0.3, 0.2, and 0.1 mgm., in 1 cc. water to which had been added toluene and chloroform also revealed no influence of the antiseptics upon the accuracy of the test.

Series VII. Elastic and connective tissue digesting enzymes.

In order to demonstrate the presence or absence of elastic or connective tissue digesting enzymes in the tubercle bacilli, an indirect method of attack had to be resorted to. In the first place it was necessary to demonstrate just how much digestion was a result of the action of the enzymes upon the proteins already obtained from the bacilli and then using this as control, to determine whether there was really any digestive action upon the elastic or connective tissues. Since the amino acid α nitrogen method of Harding and McLean was the most delicate and accurate method available for the purpose it was used as the index of the digestion taking place during the course of the experiments.

Experiment I. Elastic tissue digesting enzyme. To 10 cc. of emulsified human tubercle bacilli was added 25 cc. sterile 0.9 per cent salt solution, 5 cc. chloroform, and 10 cc. toluene. This was placed in the incubator at 37°C. for twenty-four hours when it was centrifugated and 8 cc. of the supernatant autolysate was drawn off and filtered through a sterile hard filter paper. The filtrate was divided into three equal portions, one was heated for thirty minutes in boiling water to destroy the enzymes and the other two portions were kept unheated. All were now diluted to 5 cc. and to the heated and one of the unheated portions was added 0.5 gram of elastic tissue prepared from lamb lung.[4] A definite amount of the solution (0.5 cc. each time) was withdrawn immediately, one day, three days, five days, seven days, and ten days after incubation at 37°C. and used for determining the amino acid α nitrogen content. Similarly 8 cc. of autolysate were withdrawn at four days and eight days and tested as above for digestive action upon the elastic tissue. The results of the analyses are given in the following table, the

[4] The elastic tissue was prepared by finely grinding lamb's lung and treating it at room temperature, shaking frequently, with a large amount of 5 per cent potassium hydroxide and 10 per cent acetic acid alternately until the potassium hydroxide and acetic acid solutions used remained absolutely colorless and clear after twenty-four hours contact. The elastic tissue thus prepared was washed with water until absolutely free from acetic acid and was kept for use in distilled water with toluene and chloroform as preservatives.

figures being in milligrams of amino acid α nitrogen per 0.5 cc. using asparagin as standard.

AGE OF AUTOLYSATE		DAYS					
		0	1	3	5	7	10
One day	T	0.014	0.013	0.015	0.019	0.020	0.029
	NHC	0.013	0.014	0.015	0.018	0.018	0.028
	HC	0.015	0.013	0.014	0.015	0.015	0.014
Four days	T	0.016	0.024	0.028	0.031	0.035	0.036
	NHC	0.017	0.026	0.027	0.027	0.030	0.035
	HC	0.021	0.021	0.020	0.023	0.022	0.021
Eight days	T	0.017	0.019	0.019	0.021	0.021	0.020
	NHC	0.018	0.021	0.018	0.019	0.019	0.019
	HC	0.020	0.019	0.020	0.021	0.019	0.020

T, Test, containing autolysate and elastic tissue.
NHC, Non-heated control, containing autolysate alone.
HC, Heated control, containing autolysate (heated) and elastic tissue.

Experiment II. Connective tissue disintegrating enzyme. This experiment was done exactly as in Experiment I with the exception that the 0.5 gram elastic tissue was replaced by 0.5 gram connective tissue prepared from the capsule of large tubercles.[5] The results of analyses given as the amount of amino acid α nitrogen in milligrams per 0.5 cc. of solution are tabulated in the following table.

[5] In order to prepare the connective tissue for this experiment rabbits were injected subcutaneously in the back with fat free tubercle bacilli of which 0.05 gram was used for each site of injection. In order to make a uniform suspension a 10 per cent starch paste was prepared with sterile boiling water and to every 5 cc. was added 0.05 gram tubercle bacilli. The bacilli were well mixed with the starch paste by stirring and the entire amount injected subcutaneously while warm by means of a Murphy glycerine syringe or other pressure syringe. After two to three months a large firm tubercle had formed at the site of injection and was removed. After separating all muscle, blood, and caseous material from the thick connective tissue capsule of the tubercle, the latter was well washed with physiological salt solution, then ground up fine in a meat grinder and washed 12 times with salt solution or until no more turbidity appeared in the salt solution upon vigorous shaking. The connective tissue was then heated for one-half hour at 90°C. to destroy the enzymes and again washed with salt solution. The connective tissue thus prepared was preserved for use in salt solution with chloroform and toluene.

AGE OF AUTOLYSATE		DAYS					
		0	1	3	5	7	10
One day	T	0.012	0.016	0.015	0.014	0.019	0.022
	NHC	0.012	0.011	0.013	0.019	0.021	0.021
	HC	0.011	0.012	0.013	0.012	0.013	0.013
Four days	T	0.019	0.021	0.021	0.024	0.024	0.031
	NHC	0.021	0.020	0.022	0.027	0.028	0.029
	HC	0.020	0.020	0.019	0.019	0.021	0.019
Eight days	T	0.016	0.017	0.017	0.019	0.019	0.019
	NHC	0.019	0.020	0.023	0.024	0.025	0.024
	HC	0.018	0.018	0.019	0.018	0.019	0.019

T, Test, containing autolysate and tubercle connective tissue.
NHC, Non-heated control, containing autolysate alone.
HC, Heated control, containing autolysate (heated) and connective tissue.

Summary. As a result of the above experiments it may be stated that evidence of the presence of a connective tissue, or elastic tissue, disintegrating enzyme in the tubercle bacillus was not obtained, at least by the methods used for this purpose.

COMPLETE SUMMARY

1. Tubercle bacilli of both the human and bovine varieties possess autolytic enzymes, as indicated by the non-coagulable nitrogen and amino acid α nitrogen liberated at incubator temperature after the bacilli have been killed by toluene and chloroform.

2. The bacilli themselves, or autolysates therefrom, also possess a trypsin-like enzyme capable of splitting proteins in alkaline solution, an erepsin-like enzyme capable of decomposing peptone in acid solution, a weak pepsin-like enzyme capable of splitting proteins in acid solution, a nuclease capable of splitting nucleic acid and a urease capable of decomposing urea.

3. The tubercle bacilli, or autolysates therefrom, do not possess enzymes capable of hydrolyzing starch or inverting sucrose, demonstrable by the delicate Lewis and Benedict picramic acid method.

4. Autolysates from tubercle bacilli do not possess enzymes capable of digesting elastic tissue prepared from lamb lung, or connective tissue prepared from tubercles, at least, as indicated by the methods used for demonstrating these enzymes.

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SOME CHARACTERS WHICH DIFFERENTIATE THE LACTIC-ACID STREPTOCOCCUS FROM STREPTOCOCCI OF THE PYOGENES TYPE OCCURRING IN MILK

J. M. SHERMAN AND W. R. ALBUS

From the Bacteriological Laboratories of The Pennsylvania State College and Agricultural Experiment Station, State College, Pennsylvania

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INTRODUCTION

Much confusion exists in bacteriological literature concerning the identity of the lactic-acid streptococcus and its relation to the other chain-forming cocci which occur in milk. It is generally considered that the true lactic-acid bacteria (variously known as *Bact. lactis-acidi*, *Streptococcus lacticus*, *B. lactici-acidi*, *Bacterium* or *Streptococcus guntheri*, etc.) comprise a definite and rather well defined group of organisms. But no sharp points of distinction have been established between these organisms and other streptococci, notably the pyogenic streptococci, which are usually, if not always, to be found in market milk.

The descriptions given for the lactic-acid streptococcus in the older publications on systematic bacteriology, such as the books by Migula (1900) and Chester (1901), could be applied equally well to the pathogenic streptococci. The same confusion is found in the more recent literature. Weigmann (1911) includes the streptococcus of bovine mammitis in the same group as the true lactic-acid organism. Ernst (1914) says that there is no known method by which the lactic-acid streptococcus and the mammitis streptococcus can be differentiated. Jordan (1915) states that "the milk streptococcus in all its properties is extraordinarily like *Streptococcus pyogenes*." Heinemann (1906) who has extensively studied the lactic-acid bacteria, concludes that

"*Streptococcus lacticus* agrees in morphological, cultural and coagulative properties with pathogenic, fecal and sewage streptococci."

Certain morphological characters are emphasized by some workers as differentiating the true lactic-acid organisms from other streptococci. It is pointed out that the cells of the milk streptococcus are usually elongated and also that the characteristic grouping is in pairs and short chains rather than in long chains such as are usually found in the true streptococci. These points cannot be used as sufficient ground for differentiation since it is well known that many cultures of the lactic-acid bacteria appear as perfect cocci, and the formation of the long chains is not uncommon. Further, the streptococci from pathological sources are frequently of the short chain type. Chain formation, cell size and cell shape are so greatly influenced by the nature of the nutrient medium that distinctions based upon them are bound to be of doubtful value.

Hastings (1911) has called attention to the fact that the lactic-acid organism when grown in litmus milk causes a complete reduction of the litmus previous to the curdling of the milk. This is not true of the streptococci in general with which, as a rule, the action on litmus is more gradual and not so complete. This character has been used by Hastings and his associates to differentiate the true lactic-acid bacteria from other streptococci which occur in milk and cheese. Unless, however, this test can be correlated with some other points of difference, we would not be justified in accepting it alone as giving a firm basis upon which to separate the different groups of milk streptococci.

From the study of a large collection of streptococci isolated from milk, infected udders, saliva and feces of cows, Rogers and Dahlberg (1914) were able to show that the streptococci of milk resemble those which are associated with mammitis in cattle, and that the types occurring in the cows' mouths and feces are present only in small numbers and are probably relatively unimportant in milk. They further pointed out that the streptococci from infected udders had the same physiological characteristics as the well known *Streptococcus pyogenes*. It has

recently been demonstrated (Sherman and Hastings, 1914; Evans, 1916) that a large proportion of healthy milch cows harbor streptococci within their udders which are morphologically and culturally identical with the typical *Strept. pyogenes* of mammitis and other pathological conditions. Hence such organisms are probably always present in milk produced from any considerable number of animals.

From all of these facts it would appear that there are two main groups of streptococci in milk, the true lactic-acid organisms and streptococci of the pyogenes type whose chief source is the udder. The lines of cleavage between these groups should certainly be established, and it was with the object in view of finding points of differential value that the present study was undertaken.

SELECTION OF CULTURES

The ordinary way in which to attack a problem of this kind would be to isolate a large number of miscellaneous chain-forming cocci from various grades of market milk and milk products and to subject them to a detailed study with the intention of separating them in that way into their natural divisions. Our manner of approach has been different in that the cultures were obtained in such a way as would tend to limit the collection to organisms typical of the groups under consideration, and not to include a great number of closely related types which would complicate the study and make more difficult their separation into natural groups. Since the true lactic-acid organism has not been defined so as to enable it to be distinguished from other closely related bacteria, it is obviously impossible to tell just which are and which are not typical lactic streptococci. It would seem, however, that the organisms which take an active part in the natural souring of dinorary market milk could be properly designated as true lactic-acid bacteria. Proceeding on this assumption, cultures were isolated by the following method:

Ten samples of raw milk and cream were obtained from the receiving vat of the Pennsylvania State College Creamery on as many different days. These samples were of the mixed prod-

uct from a large number of different dairy farms and so might well be considered as representative of market milk or cream of ordinary quality. After collection the milks were allowed to stand at laboratory temperature until the acidity began to rise. When the acidity reached about 0.2 per cent lactic-acid the samples were plated on lactose agar in dilutions of 1/10,000,000 cc. The use of such a high dilution should result in the isolation of the organisms which are taking the most active part in the acid fermentation. The plates were incubated at 37°C. for two days, after which small, dense colonies surrounded by a hazy precipitate[1] were fished off into sterile milk tubes. Five colonies were selected from each sample making a total of fifty cultures of this type. Without exception the bacteria selected in this way produced an acid fermentation with the formation of a smooth homogeneous curd. No consideration was given to morphology in the selection of these cultures. The cultures of this group were numbered from 1 to 50 inclusive, and after being tested for purity, were added to the collection.

Cultures representing the pyogenic type of streptococci were obtained as follows: Samples were taken from the individual cows of the Pennsylvania State College dairy herd by drawing the milk directly from the udder into sterile flasks. Samples of the mixed milk from the College herd were also obtained. This milk is of a very high grade, being produced under the best of sanitary conditions, and its flora is consequently made up chiefly of the types of bacteria which come from the udder.

These milks were plated in dilutions[2] of 1/100 on lactose agar and incubated at 37°C. for two days. Colonies similar to those of the lactic-acid organisms mentioned above were transferred to glucose broth. Cultures which formed chains in broth were

[1] Bacteriologists engaged in milk studies are familiar with the precipitate formed around the colonies of the lactic-acid bacteria when grown on agar made with Witte's peptone and containing a fermentable sugar. When grown on media made with some of the American brands of peptone this characteristic is not seen. Witte's peptone was used in the preparation of the agar from which these cultures were isolated.

[2] In the case of cow 459, which had garget, the milk was plated in a dilution of 1/1,000,000 cc.

saved for study. The fifty cultures selected came from the milks of seven individual cows and from the mixed herd milk as follows:

Culture Numbers	*From Cow*
51 to 63	663
54 to 60	337
61 to 66	459
67 to 72	631
73 to 77	734
78 to 83	620
84 to 90	624
91 to 100	mixed herd milk

All of the animals from which these samples were obtained were in a healthy condition, except cow 459, which was suffering from a case of mammitis.

MORPHOLOGY

Microscopic examinations were made of all of the 100 cultures on agar, broth and bile. Although no hard and fast rules can be given, it may be said from this study that on agar and broth the group of organisms numbered from 1 to 50 showed a greater tendency to form elongated cells than did the others. Also the typical grouping, that is the grouping of the majority of cells, was usually in pairs rather than in definite chains. But chain formation was common in these cultures, and in some of them it was the predominating grouping. The grouping in pairs was frequent among the cultures numbered from 51 to 100, but in all of these the typical arrangement was in chains.

The results obtained from growth on lactose-peptone-bile were surprising in that the lactic-acid cultures made a much more luxuriant growth than did the udder streptococci. On this medium the lactic organisms grew in long chains and appeared as typical streptococci in every way. In only one of these cultures on bile were as many cells arranged in pairs as were present in chains. This is of special interest in view of the fact that many health laboratories use bile as a presumptive test for the presence of undesirable streptococci in milk. Kinyoun and Dieter (1912) consider that the formation of chains in bile

inoculated with milk is evidence of fecal contamination, while Rogers, Clark and Evans (1916) believe from their results that it is indicative of infected udders in some of the milk producing animals. In our tests the streptococci from the udder grew more feebly in bile than did the lactic-acid streptococci, and, in most cases, could be found only with difficulty in microscopic preparations. When examined after two days incubation at 37°C. bacteria were observed in only 17 of the 50 cultures. Those seen were all in typical chain formation.

In table 1 is presented a summary of the results obtained in this study. The examinations of broth and agar cultures were made after incubation at 37°C. for one day. The growth ex-

TABLE 1

Chain formation on agar, broth and bile

	CULTURES 1 TO 50	CULTURES 51 TO 100
	per cent positive	*per cent positive*
Chains on lactose agar	58	100
Chains on glucose broth	50	100
Chains on lactose-peptone-bile	100	100
Chains predominating on broth	24	100
Chains predominating on bile	98	100

amined from agar was taken from the sloped surface, not from the water of condensation. Chain formation was not recorded as positive unless definite chains of ten or more cells in length were seen.

These data indicate that some distinction may be drawn between these two groups of streptococci based upon cell grouping. For example, all of the cultures numbered from 51 to 100 showed chains on agar and broth, while this was true of only about one-half of the organisms from the other group. If consideration is taken of the grouping of the majority of cells a greater difference is seen. On broth in all of the streptococci of the udder type the predominating arrangement was in chains whereas this was the case in only about one-fourth of the other cultures.

Although it might appear that chain formation is of some value in distinguishing the lactic-acid bacteria from other streptococci, we do not wish to make that claim. Such a differentiation would at best be a doubtful one. The method used in collecting cultures for this work was such as would tend to introduce an error in this phase of the study; only cultures which showed typical chain arrangement were selected as representing the udder-type of streptococcus, while no attention was paid to morphology in selecting the lactic-acid type. As was noted before, chain formation is not a very constant character. In this study there was apparently no correllation between the lactic cultures which grew in chains on broth and those which

TABLE 2

Action on milk

	CULTURES 1 TO 50	CULTURES 51 TO 100
	per cent positive	*per cent positive*
Milk curdled	100	100
Milk curdled in twenty-four hours	94	20
0.75 per cent lactic acid in milk	80	8

did so on agar, and it is doubtful if they would show much constancy in this respect on the same medium.

ACTION ON MILK

The amount of acid produced in milk, the presence of coagulation and the time required for curdling were noted. All of the cultures produced sufficient acid to cause the coagulation of milk when tested at 37°C. As may be seen from table 2, the organisms of the first group (cultures 1 to 50) showed a more prompt clotting of milk than did those of the second group. At 37°C. all but 3 in the first group curdled milk within twenty-four hours, while only 10 of the other group acted so promptly.

The maximum acidity produced in milk was tested by incubating the cultures for ten days at 35°C. The acidity developed

at this temperature would not be so high as if a lower incubation temperature were used, but for comparing the two classes of organisms it should serve the purpose. By this test it was found that the lactic-acid type of streptococci (cultures 1 to 50) as a group produced considerably larger amounts of acid in milk than the udder cultures. If an arbitrary standard of 0.75 per cent lactic acid is taken (table 2) it will be seen that the two groups are divided quite well by the acid producing powers of

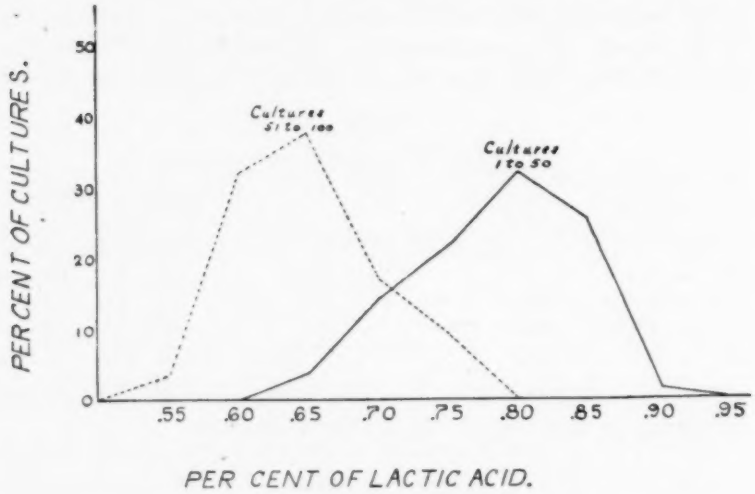

FIG. 1. FREQUENCY CURVE SHOWING ACID PRODUCTION IN MILK

their members. The difference in acid production is better shown by the frequency curves presented in figure 1.

These curves show a marked difference in the modes of the two groups as to acid production in milk. The same thing is true with respect to acid formation in lactose-peptone-bile. As was noted before, the lactic cultures appeared to grow more vigorously in bile than did the other streptococci, and the difference in the amounts of acid formed by the two types in this medium gave further evidence of that fact.

Although these results would appear to indicate that the true lactic-acid organisms as a class are capable of enduring a higher degree of acidity and of producing a more rapid coagulation of milk than the pyogenic streptococci, it is desirable to qualify this conclusion. The manner of securing the lactic streptococci was such as would tend to result in the collection of strains which were especially vigorous in their growth and action on milk. As is well known, the amount of acid produced

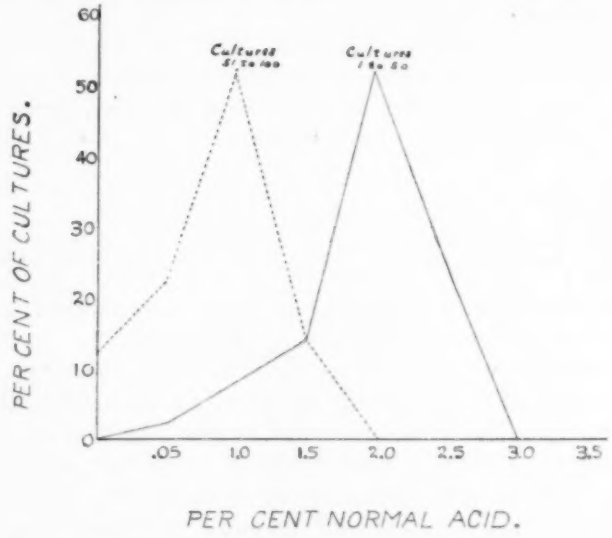

FIG. 2. FREQUENCY CURVE SHOWING ACID PRODUCTION IN LACTOSE-PEPTONE-BILE

in milk is not a very stable character and may be greatly altered by various factors such as, for example, growth on laboratory media. However, the degree of titratable acidity developed is probably of some differential value and it is not unlikely that determinations of actual hydrogen-ion concentrations, such as were made by Clark and Lubs (1915) with the colon-aerogenes bacteria and by Ayers (1915) with pathogenic and non-pathogenic streptococci, would reveal a fundamental difference between these two groups.

FERMENTATIVE CHARACTERISTICS

The value of fermentation tests with carbohydrates and related compounds in systematic bacteriology has become well established. The fermentative characteristics have been made use of especially in studies on streptococci, and by their means this very evasive group has been subdivided into a number of fairly well defined types. These tests have not been used, successfully at least, to distinguish between streptococci of the pyogenic and lactic-acid types.

In this study glucose, galactose, levulose, maltose, lactose, sucrose, raffinose, dextrin, inulin, starch, glycerine, mannit and salicin were used. The medium used for the fermentation tests had the following composition:

	per cent
Beef extract	0.3
Peptone	1.0
Dibasic potassium phosphate	0.5
Test substance	1.0

In the tests made with glucose, galactose, levulose and maltose the dibasic potassium phosphate was omitted.

In making the tests the cultures were incubated at 33°C. and then titrated against $\frac{N}{20}$ NaOH, with phenolphthalein as indicator, and the results expressed as per cent of normal acid. An increase above the control tube of 1 per cent normal acid was regarded as a positive test for fermentation. The sugars were incubated seven days and the other compounds two weeks before titration. In cases in which less than 25 per cent of the organisms were either positive or negative the minority cultures were retested. The results of this study are summarized in table 3.

A review of these data shows that the fermentative properties of the two groups are, generally speaking, quite similar. Although resembling each other on the whole, a very noticeable break in the similarity is found in the case of sucrose. It is seen that 76 per cent of the udder organisms attacked this substance as against only 6 per cent of the other group. The fermentative powers of the cultures are presented graphically in figure 3.

In this graph only the test substances are given which showed a difference of at least 5 per cent between the two groups.

From this it is evident that aside from sucrose none of the substances show sufficient difference to be of differential value. Exception might be taken to this statement in the case of maltose, in which all of the udder streptococci reacted positively while about one-fourth of the cultures of the other group were negative, but this could hardly be considered of great value for purposes of identification. Though none of the udder cultures of this collection fermented mannit, that fact is probably of no

TABLE 3

Fermentation of test substances

	CULTURES 1 TO 50	CULTURES 51 TO 100
	per cent positive	*per cent positive*
Glucose	100	100
Galactose	84	100
Levulose	94	100
Maltose	76	100
Lactose	100	96
Sucrose	6	76
Raffinose	0	0
Dextrin	8	18
Inulin	0	0
Starch	0	2
Glycerine	0	8
Mannit	28	0
Salicin	28	16

significance from the point of view of classification because it is well known that the ability to attack this substance is not uncommon among streptococci of the pyogenes type. In the work of Rogers and Dahlberg (1914) about one-fourth of the udder streptococci (from infected udders) fermented mannit, and this character was also common among cultures from other sources.

Though having no relation to the purpose of this paper, it is interesting to note that among the udder organisms all of the six cultures from one cow (no. 631) fermented salicin while only

two cultures of the remaining forty-four had that property. Of greater interest perhaps is the fact that five of the six cultures taken from cow 459, the only animal which had an infected udder, fermented dextrin. Whether this was merely a coin-

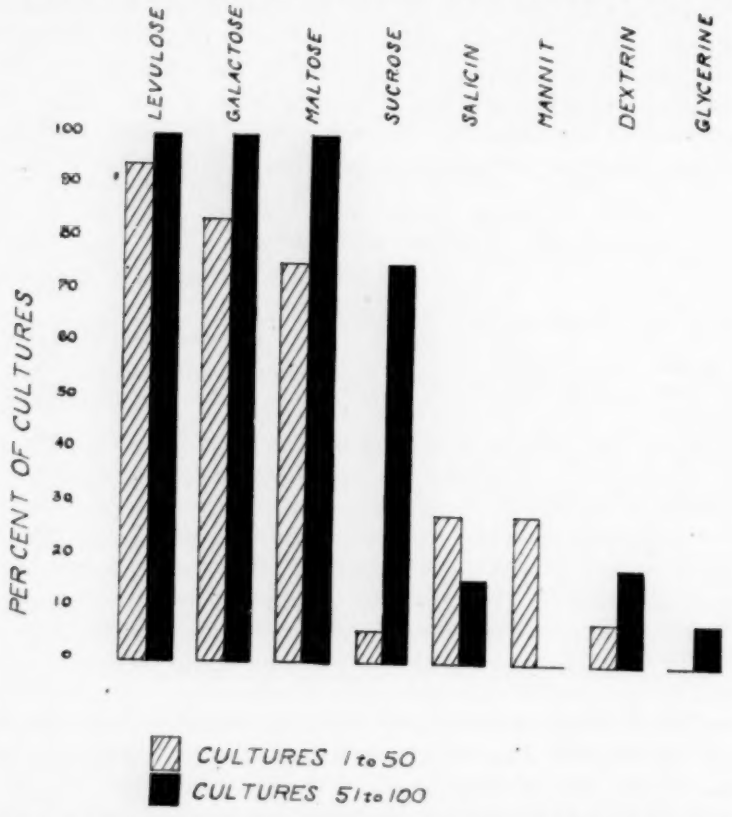

FIG. 3. GRAPH SHOWING DIFFERENCES IN FERMENTATIVE PROPERTIES OF CULTURES STUDIED

cidence or whether there exists a relation between the ability to attack dextrin and pathogenicity would require further study to answer. Two of the udder cultures failed to ferment lactose. Lactose negative streptococci have been shown by Andrewes

and Horder (1906) and by Winslow and Palmer (1910) to be numerous in the intestinal contents of the horse. In the present case, the two cultures in question not only caused an acid fermentation in milk, but produced such a vigorous one as to coagulate the casein. It seems, therefore, that these organisms were able to ferment lactose under favorable conditions but could not attack it for some reason when growing in the nutrient broth employed for the tests. A similar idiosyncrasy is noted in a few of the cultures reported by Rogers and Dahlberg (1914).

If it is conceded that the organisms of this collection numbered from 1 to 50 are representative of the group of true lactic-acid streptococci some significant points are brought out. It is generally stated in bacteriological texts[3] that the lactic organism ferments sucrose, while the statement is also found that mannit is one of the substances attacked, but according to our results the group is typically negative with both of these substances though some strains can ferment them. The typical *Strept. lacticus*, it would appear, ferments glucose, galactose, levulose, maltose and lactose. Of the cultures which failed to ferment either levulose or galactose none attacked maltose. Among the fifty cultures used there were fourteen which acted upon mannit and a similar number which fermented salicin. Nine of these attacked both compounds, thus showing quite a marked correlation between the fermentation of mannit and salicin. In most cases the cultures which fermented sucrose or dextrin also attacked both mannit and salicin.

TEMPERATURE RELATIONS

It is generally thought that the true lactic-acid streptococcus grows better at a lower temperature than the optimum for most pathogenic bacteria. Although it has not been demonstrated with any considerable number of cultures, the statement is usually made that the lactic organism has an optimum temperature of from 30°C. to 35°C. Stowell, Hilliard and Schlesinger

[3] See Weigmann's Mikologie der Milch; Marshall's Microbiology and Buchanan's Household Bacteriology.

(1913) have shown that streptococci from milk are, as a rule, more active at 20°C. than those isolated from the human throat.

Experiments at low temperatures of incubation were conducted, by inoculating litmus milk with the cultures and incubating at the desired temperature. Growth was determined by the presence or absence of visible changes in litmus milk. At 10°C. the two groups were differentiated perfectly; all of the cultures numbered from 1 to 50, which were supposed to represent the *Strept. lacticus* type, grew, while none of the group representing the *Strept. pyogenes* type did. The latter showed no change in the litmus milk after six weeks, but when put in a 37°C. incubator at the end of this period the tubes all turned acid, thus indicating that the cultures were alive but their

TABLE 4

Temperature relations

	CULTURES 1 TO 50	CULTURES 51 TO 100
	per cent positive	*per cent positive*
Growth at 10°C.	100	0
Growth at 43°C.	6	82

growth had been inhibited. The lactic streptococci all showed visible signs of growth within one week at 10°C.

Tests similar to the above were also made with high temperatures of incubation. At 43°C. was found a temperature which separated the two groups quite well, but the separation was not so perfect as at 10°C. In this case it was the pyogenic type which grew and the lactic type which failed to grow.

REDUCTION OF DYES

The use of stains as an aid in the identification of bacterial groups is not uncommon. Some familiar examples are the employment of neutral red, fuchsin and brilliant green for differentiating members of the colon-typhoid group of organisms. The reduction of neutral red was one of the characters advocated by Gordon (1905) as being of value in the separation of strep-

tococci. Rogers and Davis (1912) considered the reduction of this compound of differential value in their study of the lactic-acid bacteria of milk. As was noted earlier, Hastings (1911) has used the action on litmus in milk to distinguish the lactic-acid organism from other streptococci.

A preliminary survey of the various stains indicated that methylene blue, neutral red, litmus and indigo carmine might be of service in the present work. According to Fred (1912), bacteria, at least those types common in milk, reduce stains more actively in a milk medium than in broth, and a few tests made at the beginning of this study verified this conclusion. Tests were made by adding the dye to sterilized whole milk, the advantage of unskimmed milk being that the fat forms a layer over the surface which excludes the air quite effectively and thus reduction is not hindered. The litmus milk was prepared in the ordinary way by adding sufficient litmus solution to the milk to give a rather dark lavender color and then sterilizing. The other dyes were made as follows:

Methylene blue

Medicinal methylene blue	0.5 gram
Distilled water	1000 cc.

Indigo carmine

Indigo carmine (Kahlbaum's)	1.0 gram
Distilled water	1000 cc.

Neutral red

Neutral red (Grübler's)	0.1 gram
Distilled water	1000 cc.

The stain solutions and milk were sterilized separately and then mixed in the proportion of 1 cc. of stain to 10 cc. of milk.

In making the tests twenty-four hours old cultures of the organisms in milk were used to inoculate from, and the stain culture so prepared was incubated at 37°C. Observations were then made on three points, (1) reduction of stain, (2) time required to reduce, and (3) whether reduction was before or after curdling of milk. When no reduction was evident the cultures were allowed to remain six days before final examination was made.

As may be seen from table 5 the reduction of dyes proved to be an efficient test to differentiate between these two groups of bacteria. Methylene blue differentiated the groups perfectly, all of the true lactics causing a complete reduction of the stain within twenty-four hours and previous to curdling, while the udder streptococci failed entirely to cause reduction. After six days the tubes containing the latter class were not curdled and there was no evidence that growth had taken place. Some of these cultures were transferred to plain milk tubes but no growth occurred, thus indicating that not only were they unable

TABLE 5

Reduction of dyes

	CULTURES 1 TO 50	CULTURES 51 TO 100
	per cent positive	*per cent positive*
Methylene blue	100	0
Methylene blue (within twenty-four hours)	100	0
Methylene blue (before curdling)	100	0
Litmus	100	100*
Litmus (within twenty-four hours)	100	0
Litmus (before curdling)	100	0
Indigo carmine	100	100*
Indigo carmine (within twenty-four hours)	100	0
Indigo carmine (before curdling)	100	0
Neutral red	92	0
Neutral red (within twenty-four hours)	84	0
Neutral red (before curdling)	4	0

* Reduction not complete.

to reduce methylene blue, but that, in the concentration used, it had entirely inhibited growth and led to their destruction. The longest time required for any of the lactic-acid streptococci to reduce was ten hours, while a large majority caused a complete decolorization within eight hours after inoculation.

The actions on litmus and indigo carmine were apparently identical. In both cases the lactic-acid streptococci caused a prompt reduction; with litmus the longest time taken was eleven hours, while with indigo carmine thirteen and one-half hours was the longest time required. The udder cultures, on

the other hand, caused a reduction but it did not take place until after two or more days and then it was never absolutely complete. The important distinction, however, is that the reduction in the lactic cultures took place previous to curdling, whereas the pyogenic streptococci caused no reduction until after coagulation. This we believe represents a fundamental physiological difference between the two groups and it is to be considered, therefore, of value as a differential test. Since the litmus, indigo carmine, methylene blue and low temperature tests are all perfectly correlated, it would appear that the method of distinguishing between lactic-acid organisms and other milk streptococci based upon whether they cause reduction in litmus milk before curdling is sound.

Neutral red, which has been used considerably in studying streptococci, proved the least valuable of the four stains employed. Though the two groups were separated quite well, the distinction based upon this dye was not a perfect one. The lactic-acid organism did not cause as prompt reduction with neutral red as with the other stains, it requiring from twelve to fifty hours to decolorize this compound; and the reduction took place in most cases after curdling. Only four of the lactic cultures failed to reduce while all of the other streptococci gave negative reactions.

DISCUSSION

From the data which have been presented, it seems justifiable to conclude that the organisms studied represent two classes of streptococci; cultures numbered 1 to 50 the true lactic-acid streptococcus, and the other group (cultures 51 to 100) the pyogenic type. A review of the tests made will show a number of characters which differentiate the cultures into those two main types. The diagram given in figure 4 shows very clearly some of the fundamental differences between these groups. In this graph are presented only those tests which appeared to be of considerable differential value, those showing only slight differences between the two types being omitted.

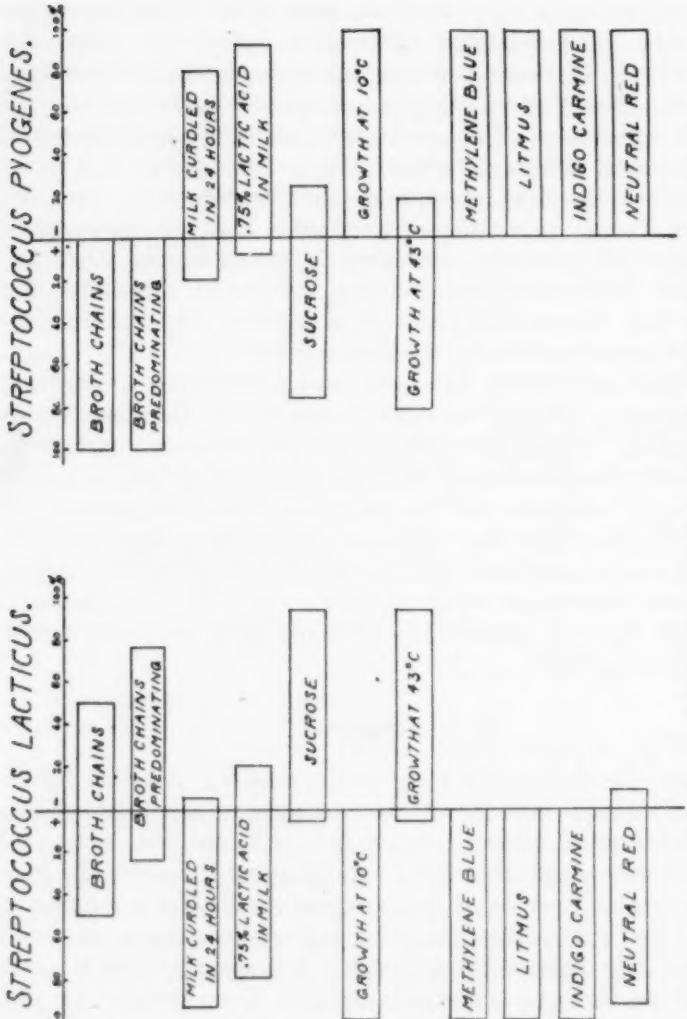

FIG. 4. DIAGRAM SHOWING DIFFERENCES BETWEEN THE LACTIC-ACID AND PYOGENIC STREPTOCOCCI

These tests, it is thought, are of sufficient value to make possible the separation of these two groups of streptococci, but it would be advantageous further to limit the number of tests and to establish the most constant ones. The differences in the groups relative to chain formation and action on milk are, in all probability, significant, but for reasons given earlier in this paper, it is not desirable to emphasize them greatly. The remaining characteristics, however, we believe to be stable ones —as stability occurs in the physiological characters of bacteria. Among the dyes neutral red did not give an absolute differentiation of the cultures studied and so might be eliminated in favor of methylene blue. As the action on indigo carmine was identical in all cases with that on litmus, it also might be discarded. The five remaining tests we wish especially to recommend as furnishing a ready and simple means of separating the milk streptococci. Growth at 10°C., reduction of methylene blue in milk, and the reduction of litmus (or indigo carmine) in milk previous to curdling are characters which have divided perfectly, in our study, the two types; *Strept. lacticus* having in all cases reacted positively while the *Strept. pyogenes* cultures, without exception, failed so to act. Absolute differentiation was not obtained with the other two tests—growth at 43°C. and the fermentation of sucrose—but the *Strept. pyogenes* type, in the great majority of cases, gave positive reactions whereas the lactic-acid streptococci in both instances were usually negative.

Although the object of this work was only to establish points of difference between the two groups of organisms, it should, aside from its main purpose, be of value in helping to define more clearly the characteristics of the *Strept. lacticus* (*Bact. lactis-acidi*) group of bacteria. As was pointed out previously, our results do not substantiate some of the generally accepted ideas concerning its fermentative properties. The facts established with reference to its temperature requirements and reducing ability, should so identify the true lactic-acid organism as to enable more reliable work than has been possible in the past concerning morphology, physiology, natural habitat and pathogenicity.

SUMMARY

A study was made of 100 cultures of organisms isolated from milk. The collection was so made that 50 of these cultures, it is believed, represented the true lactic-acid streptococcus and the other 50, streptococci of the pyogenes type.

Morphological observations were made from agar, broth and bile. The tendency to form chains on agar and on broth was not so marked among the cultures of the *Strept. lacticus* group as among the organisms of the *Strept. pyogenes* type. On lactose-peptone-bile, however, the *Strept. lacticus* cultures grew readily and formed long, typical streptococcic chains.

Among the cultures studied, the representatives of the *Strept. lacticus* type as a class had a more vigorous action on milk than did the other streptococci; coagulation was usually more prompt and larger amounts of acid were formed.

The fermentative characteristics of the two groups were quite similar with all of the substances used except sucrose. This compound was attacked by 38 of the 50 cultures of the pyogenic streptococci, while all but three of the lactic-acid streptococci failed to ferment it.

At 10°C. all cultures of the lactic-acid bacteria grew while none of the cultures of the *Strept. pyogenes* type were able to develop at this temperature. At 43°C. only 3 of the lactic organisms grew, whereas the pyogenic streptococci developed in 84 per cent of the cases.

The reduction of stains proved a valuable means of distinguishing these groups. With methylene blue all of the lactic streptococci caused reduction, while all of the pyogenic streptococci failed to reduce. Litmus and indigo carmine in milk were completely reduced before curdling by all of the *Strept. lacticus* cultures; the cultures of *Strept. pyogenes* caused no decolorization of these compounds previous to curdling, and the reduction after coagulation was slow and never absolutely complete. Neutral red, though of value, did not give as perfect a differentiation between the two types as did the other dyes.

Differences in chain formation and action on milk are doubtless of some importance, but differentiation based on these characters is probably not to be recommended. The reduction of stains, the fermentation of sucrose and the temperature tests, on the other hand, are believed to represent more constant characters and to offer means by which these two groups of streptococci may be differentiated.

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STUDIES IN THE NOMENCLATURE AND CLASSIFICATION OF THE BACTERIA

V. SUBDIVISIONS AND GENERA OF THE SPIRILLACEAE, AND NITROBACTERIACEAE

R. E. BUCHANAN

From the Bacteriological Laboratories, Iowa State College, Ames, Iowa

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Family III. **Spirillaceae** Migula, 1894, p. 237

Synonyms:

Spirillina Perty, 1852, p. 179
Spirillineae Cohn, *in ed.* Rabenhorst, 1865, p. 70
Spirobacteria Cohn, 1872, p. 180
Vibrioneae Trevisan, 1879, p. 139
Spirobacteriaceae Hueppe, 1886, p. 143
Spirilleae De Toni and Trevisan, 1889, p. 1006

Cells elongate, not spherical, bent in the form of a spiral or a segment of a spiral. Usually motile by means of polar flagella. Without sulfur granules or bacteriopurpurin.

Perty (1852) subdivided the *Spirillina* into the genera *Spirillum* and *Spirochaeta*.

Cohn (1872) likewise divided the tribe *Spirobacteria* into *Spirillum* with cells rigid, relatively short, and *Spirochaete*, with cells flexuous and relatively long. In 1875 Cohn described the additional spiral genus *Myconostoc* in which the spiral cells occurred embedded in a mass of gelatin, and included *Vibrio*, *Spirillum*, *Spirochaete* and *Myconostoc* in the algal tribe *Nematogenae*. The genera may be separated by the following key.

Key to genera of spiral Nematogenae Cohn

A. Cells not united by gelatin into families.
  1. Filaments short, sinuous.....*Vibrio*
  2. Filaments short, spiral, rigid.....*Spirillum*
  3. Filament long, spiral, flexible.....*Spirochaete*
B. Cells united into a glairy family.....*Myconostoc*

Zopf (1885, p. 50) differentiated *Vibrio* from *Spirillum* because the former was said to produce spores.

Hueppe (1886) further subdivided these forms on a similar basis as follows:

1. Without endospores.....*Spirochaete*
2. With endospores.
   a. Without change of cell form upon spore production. *Spirillum*
   b. With change of form upon spore production.....*Vibrio*

Winogradsky (1888, p. 105) removed the spirals containing sulphur to a new genus and gave them the name *Thiospirillum.*

Schroeter (1886) differentiates by the following key.

1. Cells stiff (not flexuous). With endogenous spores...*Spirillum*
2. Spirals flexuous.
   a. Cells known only in the form of a long flexuous spiral. Spores not known.....*Spirochaete*
   b. Cells usually only one half spiral, later growing to spirals. Arthrospores?.....*Microspira*

Migula (1894 p. 237) separated the genera of the *Spirillaceae* as follows:

I. Cells stiff, not flexuous.
   a. Cells non-motile.....*Spirosoma*
   b. Cells motile, with flagella.
      1. Cells with 1, rarely 2 or 3 polar flagella.....*Microspira*
      2. Cells with tuft of polar flagella.....*Spirillum*

II. Cells flexuous.....*Spirochaete*

Lehmann and Neumann (1896) subdivide the *Spirillaceae* as follows:

I. Cells short, slightly bent, with one or two polar flagella...*Vibrio*
II. Cells long, spirally bent, rigid, usually with a tuft of polar flagella.....*Spirillum*
III. Cells flexible, long, spiral, coiled filaments, flagella unknown. *Spirochaeta*

Blanchard (1906, p. 1) removed the genus *Spirochaeta* to the protozoa, and separated the bacterial genera as follows under the heading *Spirobacteria.*

A. Non motile ..... *Spirosoma*
B. Motile
1. Cells rigid, forming a segment of a circle or united into spirals, 1, 2 or 3 polar flagella ..... *Vibrio*
2. Cells definitely spiral:
a. With endogenous spores. Flagella not polar. *Spirobacillus*
b. With or without endogenous spores. Flagella polar *Spirillum*

The following generic names have been proposed for spiral organisms.

Spirillum Ehrenberg, 1830, p. 38
Myconostoc Cohn, 1875, p. 183
Pacinia Trevisan, 1885, p. 83
Microspira Schroeter, 1886, p. 168
Pseudospira De Toni and Trevisan, 1889, p. 1018
Euspirillum De Toni and Trevisan, 1889, p. 1009
Euspirosoma Migula, 1900, p. 955
Liquidovibrio Jensen, 1909, p. 333
Solidovibrio Jensen, 1909, p. 333
Paraspirillum Dobell, 1911, p. 94
Vibrio Mueller, 1773, p. 39
Spirodiscus Ehrenberg, 1838
Spiromonas Perty, 1852, p. 171
Spirulina Hueppe, 1886, p. 146
not *Spirulina* Turpin, 1827
not *Spirulina* Cohn, 1853
Spirobacillus Metschnikoff, 1889, p. 61
Streptospirillum Billet, 1890, p. 24
Spirosoma Migula, 1894, p. 237
Sporospirillum Jensen, 1909, p. 340

The generic name *Spirulina* is invalid because of prior use for another genus of plants.

The species of the following genera cannot be identified from the description. *Myconostoc, Spirodiscus, Spiromonas, Streptospirillum, Spirobacillus, Sporospirillum.*

The following are subgeneric terms: *Euspirillum, Euspirosoma, Pseudospira.*

The following genera are probably not invalid for any of the preceding reasons.

Liquidovibrio, Microspira, Pacinia, Paraspirillium, Solidovibrio, Spirillum, Spirosoma, Vibrio.

The following key will serve to differentiate the genera which may be recognized at present.

Key to genera of Spirillaceae

A. Cells not larger at center, not tapering.
  1. Cells usually short, only a segment of a spiral. One or rarely two or three polar flagella.....Genus 1. *Vibrio*
  2. Cells longer, usually with a tuft of polar flagella. Genus 2. *Spirillum*
B. Cells enlarged at center and tapering.....Genus 3. *Paraspirillum*

Genus 1. **Vibrio** Mueller, 1786 p. 39 emended

Synonyms

Pacinia Trevisan, 1885, p. 83
Pseudospira De Toni and Trevisan, 1889, p. 1018
Microspira Schroeter, 1886, p. 168
Liquidovibrio Jensen, 1909, p. 333
Solidovibrio Jensen, 1909, p. 333

Short, bent rods, sometimes almost straight. Motile by means of a single (rarely 2 or 3) polar flagellum. Aërobic, and facultative. Grow well on ordinary media. Frequently liquefy gelatin. Not enlarged near center. No spores. Usually gram negative.

The type species is *Vibrio cholerae.* The generic name *Vibrio* has been used in several senses by different writers. Most of the species included in this genus by writers before the time of Cohn are now definitely placed in other genera or cannot be identified. Pacini (1851) used the designation *Vibrioni of cholera,* though it is not certain that he actually saw the organism causing the disease.

The *Vibrio* of Müller was any very simple, "terete, elongate worm." Ehrenberg (1838) defined the genus to include straight flexuous rods. Cohn (1872, p. 178) emended the genus to include organisms characterized by a wavy motion of the fila-

ments, the rotation of which gives the appearance of sinuous motion, this character indicating transition to the spiral bacteria. Zopf (1885, p. 61) definitely designates the genus as spiral. Some authors retained the older idea of the genus, and introduced new generic names as *Pacinia* and *Microspira*, others used the generic name *Vibrio* for short spirals. The latter conception is held by Loeffler, Fischer, Lehmann and E. F. Smith and is the one adopted here.

Vuillemin (1913, p. 518) concludes that *Vibrio* used as a generic name should be suppressed because of its varied meanings. He would use the generic name *Microspira.* However, if *Vibrio* should be abandoned, *Pacinia* Trevisan would definitely have priority.

Genus 2. **Spirillum** Ehrenberg 1830, p. 38 emended, Migula, 1894, p. 237.

Synonym:

Spirosoma Migula, 1894, p. 237

Cells definitely spiral, not enlarged at center, motile by means of 5 to 20 polar flagella, or non-motile. Not readily cultivated in ordinary culture media.

The type species is *Spirillum undula* (Müller) Ehrenberg.

Genus 3. **Paraspirillum** Dobell 1911, p. 99

Cells spiral or S shaped, like spirilla, variable in thickness, with a well marked thickening toward middle of the body, and tapering toward the ends, a much elongated and spirally twisted spindle. Motile by means of flagella.

The type species is *Paraspirillum vejdovskii* Dobell.

Family IV. **Nitrobacteriaceae** Fam. nov.

Cells spherical or rod shaped, motile or non-motile, not growing on ordinary laboratory media in the presence of organic matter. Securing growth energy primarily by the oxidation of ammonia to nitrites or of nitrites to nitrates.

The genera may be differentiated by the following key:

Key to the genera of Nitrobacteriaceae

A. Cells rod shaped.
1. Oxidizing ammonia to nitrous acid. Motile. Genus 1. *Nitrosomonas*
2. Oxidizing nitrous acid to nitric acid.....Genus 2. *Nitrobacter*
B. Cells spherical.....Genus 3. *Nitrosococcus*

Genus 1. **Nitrosomonas** Winogradsky, 1892, p. 127

Synonym:

Nitromonas Winogradsky

Cells rod shaped. Motile. Not growing readily on organic media. Oxidizing ammonia to nitrates.

The type species is *Nitrosomonas europoea* Winogradsky.

Genus 2. **Nitrobacter** Winogradsky, 1892, p. 127

Cells rod shaped, non-motile, not growing readily on organic media, oxidizing nitrates to nitrates.

Winogradsky named no species, although one was described. It may be termed *Nitrobacter winogradski* and regarded as the type species.

Genus 3. **Nitrosococcus** Winogradsky, 1892, p. 127

Cells spherical. Not growing readily on organic laboratory media. Oxidizes ammonia to nitrites.

Winogradsky termed the single species "les microbes nitreux de nouveau monde" but without a species name. It may be termed *Nitrosococcus americanus* and regarded as the type.

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THE DIRECT OR BREED METHOD FOR COUNTING BACTERIA IN TOMATO CATSUP, PULP OR PASTE

CHARLOTTE VINCENT

Baltimore City Health Department, Baltimore, Maryland.

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The method used for counting bacteria in tomato catsups, pulp or paste depends on the use of the Zeiss blood counter. In this method a drop of catsup diluted with two portions of distilled water is placed in the counting chamber so as to cover the space within the moat. This stands for fifteen minutes before counting and the bacteria in 25 small squares are then counted and an average is obtained for 5 of these small squares. The result is then multiplied by 2,400,000 to find the number of bacteria per cubic centimeter. This method is described by B. J. Howard (1911) in detail

In counting the number of bacteria per cubic centimeter in a number of samples of catsup I found that this method was not entirely satisfactory, as the bacilli are hard to differentiate from micrococci or various small particles found in the catsup. Micrococci are not counted according to this method as they are so liable to be confused with other bodies such as particles of clay.

It was thought that these sources of error might be overcome by the use of the direct method of counting bacteria devised by Prescott and Breed (1911), and the same technique was used as that described under the "Microscopic Method of Analysis" in Standard Methods of Bacteriological Analysis of Milk. (A. P. H. A., 1916). The catsup is diluted with two parts of sterile water, since this dilution has proven satisfactory in counting most specimens when the Zeiss counting chamber is used. With a sterile pipette calibrated to deliver 0.01 cc. the diluted catsup is deposited on a second glass slide and evenly spread over an area of 1 sq. cm. by means of a sterile needle. After drying, the

ZEISS COUNT			DIRECT COUNT		
Sample	Average per square	Bacilli per cubic centimeter		Average field	Bacilli per cubic centimeter
I	13	31,200,000	Tomato catsup	188	56,400,000
	19	45,600,000		172	51,600,000
II	12	28,800,000	Tomato catsup	392	117,600,000
	10	24,000,000		312	93,600,000
III	30	72,000,000	Tomato catsup	388	116,400,000
	26	62,400,000		324	97,200,000
IV	18	43,200,000	Tomato pulp	480	144,000,000
	22	52,800,000		482	144,400,000
V	16	38,400,000	Tomato pulp	254	76,400,000
	12	28,800,000		200	60,000,000
VI	30	72,000,000	Tomato pulp	840	252,000,000
	26	62,400,000		844	253,200,000
VII	21	12,600,000	Tomato catsup	33	9,900,000
	20	48,000,000		45	13,500,000
VIII	16	38,400,000	Tomato pulp	56	16,800,000
	24	57,600,000		60	18,000,000
IX	19	45,600,000	Tomato catsup	92	27,600,000
	16	38,400,000		92	27,600,000
X	28	67,200,000	Tomato pulp	304	91,200,000
	18	43,200,000		352	106,000,000
XI	30	72,000,000	Tomato pulp	308	92,400,000
	18	43,200,000		328	98,400,000
XII	50	120,000,000	Tomato catsup	272	81,600,000
	40	96,000,000		192	57,600,000
XIII	18	43,200,000	Tomato catsup	384	115,200,000
	20	48,000,000		478	143,600,000
XIV	30	72,000,000	Tomato catsup	124	37,200,000
	36	86,400,000		84	25,200,000
XV	46	110,400,000	Tomato catsup	852	255,600,000
	42	100,800,000		796	238,800,000

slide is immersed in 95 per cent alcohol for one minute to fix the smear, dried in the air, stained with Loeffler's methylene blue for two minutes, washed off in water, dried and examined with the $_1{}^1{}_2$ inch oil immersion lens. The microscopic field is then standardized by means of a stage micrometer, with the selection of proper oculars and the adjusting of the draw tube so as to bring the diameter of the whole microscopic field to 0.205 mm. Thirty fields are counted and the average for one field is then multiplied by 3 on account of the dilution of the catsup, and this result is then multiplied by 300,000. The result represents the number of bacteria per cubic centimeter of catsup or other tomato product.

Fifteen samples of tomato catsup or pulp were counted by means of the Zeiss blood counter and by the modified direct count, two counts being done on each sample and the results being averaged The able opposite gives the results in detail, and a comparison can be made between the results obtained from the Zeiss blood counter and by means of the direct count.

CONCLUSION

An examination of the above table will show that the direct count gives much larger numbers of bacteria than the Zeiss blood counter.

Thus, the advantage of the direct count is, first, that a bacillus can always be distinguished from micrococci or inert material, while one is often in doubt when using the Zeiss counter. Secondly, that micrococci can be counted since they are easily stained and can be noted when this method is used.

It is just as important to count the number of micrococci as bacilli, since the former probably take just as active a part in the deterioration of tomato products as the latter. Micrococci are difficult to distinguish by means of the Zeiss counting apparatus but are easily recognized when the direct method is used.

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BLUE-PRINTING DIRECTLY FROM AGAR PLATES

JEAN BROADHURST

Teachers College, Columbia University

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This spring in planning, for teaching purposes, a large number of illustrative series of photographs showing comparative bacterial conditions (boiled and unboiled water, effects of disinfectants, milk kept at different temperatures, etc.) we found photographs as expensive as permanent glycerine-agar plates themselves. With amateurs the results are most uncertain, even when a really good camera can be secured; and with the delays incident to developing and printing the Petri dishes often spoil or change materially before new exposures can be made.

We finally hit upon the expedient of using blue-print paper, printing directly from the Petri dish, and omitting the camera entirely. The Petri-dish was placed flat upon the blue-print paper and then exposed to the direct rays of the sun—a method long used for leaf-prints. Occasionally the more transparent colonies were "touched up" by using a fine-tipped brush to apply thick white paint to the under side of the Petri dish. Unless the agar layer is thin and unless the light rays fall at right angles to the dish, "touched up" colonies appear blurred in the prints.

The blue print paper used by architects is sensitive enough for most purposes, and much cheaper than the kind sold by photographic supply houses. We had the large sheets cut into 5-inch squares, the total cost (including the cutting) totalling but $1.65 for 500 squares.

A large board was used for holding the Petri-dishes. To the board were fastened by screws or matting tacks narrow strips of metal (old jig-saw blades, in this case). When these were lifted or swung into position (2 or 3 to a dish) their free tips pressed

upon the edge of the Petri dish and held it firmly against the paper.

Blue-print paper has been used for photographing photogenic bacteria, but this particular application is perhaps new. Teachers will readily recognize the value of this method in permanently recording most of the results for which Petri-dishes are used.

I had hoped to publish photographs of the prints as prepared. The blueprints, however, on account of their color, do not reproduce well enough to show their real value. "Black and white" architects' paper which could be used equally well is imported and not to be procured at this time.

COMPARISON OF LOEFFLER'S COAGULATED BLOOD SERUM AND BLOOD SERUM WITH THE ADDITION OF STERILE OX GALL

HELEN R. ODELL

New York State Department of Health, Albany, New York

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In 1913 Von Drigalski and Bierast reported on the results obtained by the comparative use of Loeffler's original coagulated blood serum plate medium and blood serum medium with the addition of sterile ox gall, in the isolation of the diphtheria bacilli. Drigalski's medium was prepared according to the original formula with the addition of 0.13 parts ox gall. They concluded from their work that the modified Loeffler medium rendered the isolation of diphtheria bacilli more certain and in many cases easier, due to the increase in the number of diphtheria colonies on the plates.

Büsing in 1914 repeated the work and compiled the results of various other workers in the use of Drigalski's serum medium. The following table shows the results collected by Büsing.

Table of results

WORKERS	POSITIVE ON BOTH MEDIA	POSITIVE ON DRIGALSKI MEDIA ONLY	POSITIVE ON LOEFFLER MEDIA ONLY
Drigalski and Bierast, 1913	36	17	0
"Halle"	81	17	0
Seligman	30	9	3
Voelckel, 1913	35	0	4
Schulz, 1913	8	0	2
Grundmann, 1913	70	0	20
Büsing, 1913	79	2	7

There were 384 positive findings with Drigalski's medium and 375 with Loeffler's medium, showing in 420 examinations

only 9 cases in which the Drigalski medium proved more advantageous.

The original work which was carried on in this laboratory in the early part of 1916 consisted in the inoculation, with swabs received with the routine throat cultures, of Loeffler's coagulated serum medium in slants and the simultaneous inoculation of slants of bile serum medium made according to the original formula of Drigalski and Bierast (1913). In this way 400 comparative examinations with the bile and the Loeffler's media were made. One hundred and six examinations gave positive findings on both media. Positive results were obtained on Loeffler's medium only, in 8 examinations, and on the bile media alone, in 4 examinations. Growth of the diphtheria bacilli was more luxuriant on the bile medium in 32 of the 106 positive examinations, but in 5 cases growth was more vigorous on the Loeffler medium.

This work was subsequently repeated, the bile-serum tubes being inoculated first with the swabs, to exclude error due to a lessening in the number of diphtheria bacilli carried over in serial inoculations. The results obtained in 78 comparative tests conducted in this manner showed essentially the same findings as in the first experiment.

Thus the results of our experiment comparing the value of bile-serum and Loeffler serum tubes are in close agreement with those of previous workers who used the plating method. In some instances, in our work, the inhibiting effect of bile upon the common organisms present in the mouth, especially the staphylococcus, was very marked; but in general the modified Loeffler's coagulated serum medium offers no advantage over the plain Loeffler's medium in the isolation and identification of *Bacillus diphtheriae*.

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